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Ecological Research Series

TEMPERATURE, INFECTIOUS DISEASES, AND THE IMMUNE RESPONSE IN SALMONID FISH

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TEMPERATURE, INFECTIOUS DISEASES, AND THE IMMUNE RESPONSE IN SALMONID FISH

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ABSTRACT

Investigations of the effect of temperature on infections of salmonid fish were conducted. Aeromonas salmonicida infection was studied in chinook salmon and steelhead trout; Aeromonas liquefaciens infection in chinook and coho salmon. In all cases mortality rates were high at 64 to 69 F; usually moderate at 54 to 59 F; and low or zero at 39 to 49 F. Progress of the infections was accelerated at higher temperatures, and retarded at lower temperature levels.

Bacterial kidney disease was studied in coho salmon and steelhead trout. The temperature range of 44 to 54 F was optimal for the development of fatal infection, as indicated by mortality rates of 78 to 100%. Higher temperatures had a suppressing effect, which was marked at 69 F.

Temperatures of 59 to 69 F were optimal for the formation of agglutinating antibody when juvenile coho salmon were injected with a killed suspension of A. salmonicida. At lower temperatures less antibody was formed, and no significant amount was produced at 39 F 60 days after injection of antigen.

Oral immunization of juvenile coho salmon with a vaccine consisting of formalin killed Vibrio anguillarum cells incorporated in their diet protected them against fatal infection when the fish were held at temperatures from 39 to 69 F during immunization.

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SECTION I

CONCLUSIONS

1. Water temperatures of 59 F and above produce a high percentage of fatal infections in juvenile coho and spring chinook salmon, and steelhead trout injected with Aeromonas salmonicida. Even at 49 and 54 F from 30 to 70% of the fish may succumb to infection.

2. Mortality rates from infection are very low among juvenile coho and spring chinook salmon and steelhead trout injected with A. salmonicida and held at temperatures of 39 and 44 F.

3. The mean time to death after injection with A. salmonicida falls in the range of 2.3 to 3.5 days for juvenile coho and chinook salmon, and juvenile steelhead trout held at 69 F. This increases progressively as water temperature decreases, resulting in a range of 18.4 to 20.3 days among these species at 44 F.

4. The percentage of fatal infections among juvenile coho and chinook salmon and juvenile steelhead trout injected with Aeromonas liquefaciens is very high at temperatures of 64 F to 74 F, moderate at 54 and 59 F, and very low or zero at 39 to 49 F.

5. The mean time to death after injection with A. liquefaciens is in the range of 1.1 to 1.7 days for juvenile coho and chinook salmon, and juvenile steelhead trout held at 69 F. At 54 F the infection develops more slowly, and the average time to death is in the range of 3.1 to 4.7 days.

6. The effect of temperature on the growth rate of A. liquefaciens in vitro appears to be similar to its effect on the rate of progress of the infection in fish.

7. When juvenile coho and spring chinook salmon, and steelhead trout are infected with Flexibacter columnaris by water contact, the percentage of fatal infections is high at temperatures of 64 to 74 F, moderate at 59 F, low at 54 F, and approaches zero at 49 F and below.

8. The mean time to death for juvenile coho and chinook salmon and steelhead trout after exposure to F. columnaris by water contact falls in the range of 1.7 to 2.5 days at 60 F. At 54 F progress of the disease is retarded, resulting in a range of 6.7 to 11.0 days.

9. A temperature of 54 F is close to the threshold for development of fatal infection of salmonids by F. columnaris. All the evidence indicates that temperatures below 54 F are required for complete suppression of the disease in salmonid populations that have been exposed to the pathogen.

10. Water temperatures in the range of 44 to 54 F appear to be optimal for the development of fatal bacterial kidney disease among juvenile coho salmon and steelhead trout inoculated with the organism. Percent mortality is high in this range, moderately high at 59 F. A temperature of 64 F is less favorable for the disease, as indicated by a significant reduction in mortality, while at 69 F only 8 to 14% of the inoculated fish develop fatal disease. It is believed these temperatures retard progress of this infection.

11. Bacterial kidney disease differs from A. salmonicida and A. liquefaciens and F. columnaris infections in the fact that it is suppressed by a water temperature of 69 F. It is a slowly progressing infection in juvenile coho salmon and steelhead trout. The mean time from inoculation until death may vary from a range of 21.7 to 33.7 days at 59 F to a range of about 61.0 to 71.8 days at 44 F. At 39 F the process is further retarded resulting in a mean time to death in the range of 87 to 98 days.

12. Agglutinating antibody against A. salmonicida produced in juvenile coho salmon in response to a single injection of the killed bacteria in Freund's adjuvant appears to be influenced by the water temperature at which the fish are held. Temperatures of 59 to 69 F are optimal for antibody production, and the highest concentrations of antibody in the blood are reached about 45 days after antigen injection. At this time less antibody is produced at 54 F and still less at 49 F. At 39 F no significant amount of antibody is formed, even after 60 days.

13. The oral administration of a vaccine consisting of formalin killed Vibrio anguillarum cells incorporated in the Oregon Moist Pellet diet to juvenile coho salmon protects these fish against fatal vibriosis when they are exposed to the infection by residence in estuarine waters containing this pathogen. Immunization appears to be effective when the fish are held at any temperature from 30 to 69 F during the 15 day period of vaccine administration. The oral vaccination does not induce the formation of circulating antibody against the bacterium.

SECTION II

RECOMMENDATIONS

In an earlier report dealing with the effect of water temperature on infectious diseases of salmonid fish (1) a major recommendation was advanced which is restated as follows: Water temperatures in many rivers of the Pacific Northwest from May through October are in a range favorable for the progress of the important infectious diseases of salmonids. During this period threshold temperatures for these diseases are reached and a maximum of 70 F is not uncommon. Temperatures favorable to the host generally occur from November through April. It is, therefore, recommended that no additional sources of heat should be allowed to enter these rivers. Added heat during the period from May through October could only serve to further enhance the severity of these diseases. Increasing water temperatures from November through April would shorten the period when conditions are most favorable for the host. The experimental evidence presented in this report confirms and strengthens our original observations and provides additional validity to this principal recommendation.

SECTION III

INTRODUCTION

The chief objectives of this project were: (a) To determine the effect of water temperature upon the mortality resulting from the more important infectious diseases of salmonid fish; and (b) To obtain additional information concerning the effect of water temperature on antibody formation and the immune response in salmonid fish.

The diseases which have been studied have included those caused by Flexibacter columnaris, Aeromonas salmonicida, Aeromonas liquefaciens, and the organism causing bacterial kidney disease (Corynebacterium sp.). In an earlier report (1) similar studies of ceratomyxosis and infectious hematopoietic necrosis were described. Fish species in the experiments reported here were juvenile coho (Oncorhynchus kisutch) and chinook salmon (Oncorhynchus tshawytscha) and steelhead trout (Salmo gairdneri).

The general experimental plan consisted of the following: (a) infection of susceptible fish species by the most appropriate method; (b) subsequent observation of these fish at one of eight temperatures in flowing pathogen free water. Eight temperatures from 39°F to 74°F, with 5° increments, were provided. For each experimental temperature, groups of 50 or more infected fish have been employed, distributed equally between 2 tanks. Parallel groups of normal uninfected fish have been held under identical conditions.

All experimental fish were observed daily for appearance of symptoms, lesions, or fatal infections. Dead fish were removed when observed, and were autopsied and bacteriological cultures prepared from appropriate organs. Observations were continued until no further deaths occurred.

The effect of the various water temperatures upon each type of infection was judged by the fraction of the group of fish held at each temperature that developed fatal infection caused by the specific pathogen, and by the mean death time for those that succumbed in each group.

SECTION IV

EQUIPMENT DESIGN AND FABRICATION PHASE

The design and fabrication of the equipment used in the project were described in an earlier report (1).

SECTION V

EFFECT OF WATER TEMPERATURE ON INFECTION OF SALMONIDS BY *AEROMONAS SALMONICIDA* AND *AEROMONAS LIQUEFACIENS*

Materials and Methods

The SS-70 BE-3 strain of *A. salmonicida*, which was used in the experiments reported here, was isolated from the kidney of a chinook salmon (*Oncorhynchus tshawytscha*) at the South Santiam Hatchery in Oregon. It was passed through a series of 13 transfers in juvenile coho salmon (*Oncorhynchus kisutch*) by intraperitoneal inoculation of a suspension of kidney tissue from the fish infected in the preceding transfer. Kidney tissue from the last fish in the series was then macerated, suspended in sterile skim milk and lyophilized.

Aeromonas liquefaciens, strain K-1, used in the experiments with spring chinook salmon, was isolated from the kidney of a shad (*Alosa sapidissima*) during an epizootic in Coos Bay, Oregon. Stock cultures were maintained on peptone beef extract glucose agar covered with a layer of neutral mineral oil. This medium contains 10 gm of peptone (Difco), 5 gm of glucose, 10 gm of beef extract, 5 gm of sodium chloride, and 15 gm of agar per liter. *A. liquefaciens* strain JZ-45 was used in the experiments with coho salmon. It was isolated from a juvenile spring chinook salmon at the OSU Fish Disease Laboratory where it appeared to be causing fatal infections in fish held at 64 and 69°F.

The salmonid fish used in the work reported here were juvenile coho and spring chinook salmon, and juvenile steelhead trout. Their average weight ranged from 18 to 35 grams in different experiments. They were generously donated for this project in relatively large numbers by the Oregon Wildlife Commission and the Fish Commission of Oregon.

Experimental infections in fish were produced by the intramuscular or intraperitoneal injection of 0.05 to 0.1 ml of a culture of the pathogen in brain heart infusion (BHI) broth or peptone-beef extract-glucose (PBG) broth suitably diluted to contain a small number of LD_{50}. Although it would have been distinctly preferable to use a more natural method for establishing infection, previous experience with *A. salmonicida* and *A. liquefaciens* indicated that injection was the only method that could be relied on to produce fatal infection in a large percentage of exposed fish. These organisms, while pathogenic for fish, do not always possess highly invasive properties.

The method used to temper fish to the various experimental water temperatures was as follows: When received from the hatchery, the fish were maintained at 54 F. At the beginning of an experiment, the fish to be used were transferred to an 18 gallon tank, and the water was gradually replaced with water at the next temperature increment, either 49 to 59 F, over a period of 1 to 1.5 hours. Fish were held at the new temperature for 48 hours before the cycle was repeated by replacing the water and achieving the next temperature increment either 44 or 64 F. This process was repeated until groups of fish had reached the assigned temperature levels covering the range from 39 to 72 or 74 F at five degree intervals.

Infections were confirmed at necropsy by streaking small fragments of kidney tissue on plates of BHI agar, which were incubated at room temperature (about 22 C). Colonies of A. salmonicida were identified by Gram stain, colony morphology, brown pigment production, and a positive oxidase reaction. Identification of colonies of A. liquefaciens was made from the cultures incubated at 37 C by agglutination with specific antiserum (A. salmonicida does not grow at 37 C).

The experimental design adopted in this work required the use of sixteen 18 gallon aquaria for each experiment. Thus two tanks were provided for each of the eight water temperatures. Eight tanks, one at each temperature, were assigned to groups of fish to be infected with the pathogen being studied, while the remaining eight were assigned to groups of uninfected control fish that received sham injections. The number of fish per tank was at least 25, and in some experiments was increased to 35. Two essentially identical experiments were conducted concurrently, each one consisting of eight groups of infected fish and eight control groups. The purpose of this plan was to provide information concerning the degree of variation to be expected between groups of fish receiving, insofar as possible, exactly the same treatment.

Experimental Phase

Effect of Temperature on Infection with *Aeromonas salmonicida*

Experiments designed to determine the effect of water temperature on experimental infection of juvenile coho and spring chinook salmon with *A. salmonicida* have been described in an earlier report (1). However irregularities in the results obtained with the chinook salmon strongly indicated that some factors other than temperature were influencing the mortality data. Accordingly these experiments were repeated and similar tests carried out for the first time in juvenile steelhead trout (*Salmo gairdneri*).

In the experiments with spring chinook salmon, 400 fish averaging 19 grams in weight, were distributed at random among 16 tanks, 25 fish per tank. Each tank contained 18 gallons of well water, flowing at a rate of 0.5 gallons per minute. Eight tanks contained fish to be infected, and eight contained fish to be used as uninfected controls. One tank in each group of eight received flowing water at 72 F, another received water at 69 F, a third received water at 64 F, and so on, so that groups of fish were maintained at each 5 degree increment of temperature from 39 to 69 F, and also at 72 F. A second identical experiment, requiring an additional 400 fish, was carried out concurrently.

Fish to be infected received an intraperitoneal injection of 0.1 ml of a 19 hour culture of *A. salmonicida* strain SS-70 BE-3 in BHI broth diluted to contain about 5400 colony forming units, or about 18 LD_{50}. Control fish received a sham injection of 0.1 ml of sterile phosphate buffered saline. After injection all groups of fish were held at their respective temperature for 13 days. The experiments were terminated at that time due to a disease condition referred to as tail rot in both infected and control groups at 54 F and below. Dead fish were collected daily, autopsied and cultures prepared by smearing kidney tissue on plates of BHI agar. After termination, all surviving fish in infected groups of 5 controls from each temperature group were autopsied and cultured. *Aeromonas salmonicida* colonies were identified by Gram stain, colony morphology, brown pigment production, and a positive oxidase reaction.

Results of the two experiments are shown in Table 1. Among the infected groups the mortality exceeded 90% at 69 and 72 F, and in most cases decreased significantly with each 5 degree reduction in temperature. Thus it was lower at 64 than at 69 F, at 59 than at 64 F, at 49 than at 54 F, and at 44 than at 49 F. The data show that the development of fatal infection in juvenile chinook salmon due to *A. salmonicida* was suppressed at water temperatures of 39 and 44 F, and enhanced progressively at temperatures of 49, 54, 64 and 69 F. These results are very similar to those obtained in comparable experiments with juvenile coho salmon (1).

Table 1. Effect of water temperature on Aeromonas salmonicida infection in juvenile chinook salmon.[a]

Water temperature[c]	Fraction of each group that died				Percent mortality; 2 expt. combined[d]		Mean time from infection to death in days
	Experiment 1		Experiment 2				
	Infected[b]	Controls	Infected[b]	Controls	Infected[b]	Controls	
72 F	22/23	0/23	20/20	0/24	98	0	2.2
69 F	22/22	2/22	20/23	0/25	93	4	2.3
64 F	18/25	2/25	19/25	3/25	74	10	3.4
59 F	13/25	3/25	15/25	2/25	56	10	5.9
54 F	16/25	5/25	19/25	3/25	70	16	6.9
49 F	12/25	2/25	12/25	1/25	48	6	9.4
44 F	4/25	4/25	9/25	0/25	26	8	12.2
39 F	2/25	0/25	2/25	3/25	8	6	n.d.[e]

[a]The average weight of the fish at the beginning of the experiment was 19 gm.

[b]Fish were infected by an intraperitoneal injection of 0.1 ml of a 19 hour culture of A. salmonicida strain SS-70 BE-3 in Brain Heart Infusion broth, diluted to contain about 5400 colony forming units, or about 18 LD_{50}. Control fish received a sham injection of 0.1 ml of sterile phosphate buffered saline.

[c]All groups of fish were held at the indicated temperatures for 15 days. The experiment was terminated at that time due to extensive tail rot in both experimental and control groups at 54 F and below. Dead fish were collected daily, autopsied, and cultures made from kidney tissue. After termination, all surviving experimental fish and 5 controls from each temperature group were autopsied and kidney cultures prepared.

[d]The least significant difference between percent mortality values was determined to be 13.82% at the 0.05 probability level (Appendix, page 58).

[e]n.d. indicates no data.

The results of bacterial cultures of kidney tissue from the infected groups of fish appear in Table 2. Aeromonas salmonicida was recovered from 88 to 100% of the mortalities in each experimental group, and these findings provided confirmatory evidence as to the cause of death. The organism was not recovered from any control fish examined. It is of interest to note that the bacterium was also recovered from a considerable number of the fish that survived at temperatures of 49 F and below. This was not the case in the coho salmon experiments (1). It seems probable that the persistence of the bacteria in survivors may be related to the relatively short duration of the chinook experiments; i.e. 13 days compared to 55 days in the case of the coho. The longer holding period might have provided the time necessary for the defense mechanisms of the surviving fish to clear the invading bacteria from the tissues.

A linear relationship between the log of the number of days to death and water temperature was observed and confirmed by regression analysis (Fig. 1). The correlation coefficient was -0.8607 and was found to be highly significant (Appendix, page 65). The R^2 value of 0.7407 indicates that time to death was about 74% dependent on temperature. The mean values decreased from 12.2 days at 44 F to 2.3 days at 69 F. Thus the progress of the fatal infection in these fish was accelerated at the higher temperatures and retarded at the lower temperatures. Values for the mean time to death were computed on the basis of the fish that died during the course of an experiment, rather than the total number of fish in the test group. These values were calculated as the geometric means of the individual times to death in days.

The effect of temperature on infection of juvenile steelhead trout by A. salmonicida was studied in duplicate experiments carried out concurrently. The fish averaged 35 gm, and the infecting dose of the bacterium contained about 600 colony forming units, or about 2 LD_{50}. In this case all experimental groups of fish were held at their respective temperatures over a period of 33 days.

The results obtained are presented in Table 3. The highest water temperature in these experiments was 74 instead of 72 F, which proved to be too warm for the uninfected control fish, as 96% of them died within the first few days. At 69 F however only 10% of the controls died, compared to 96% of the infected groups. Among the latter the percent mortality decreased with decreasing water temperature from 84% at 64 F to 30% at 54 F, 10% at 44 F, and 2% at 39 F. As in the chinook salmon experiments, fatal infection was suppressed at 39 and 44 F, and progressively enhanced as the temperature increased from 49 to 69 F.

Table 4 contains the results of bacterial cultures of kidney tissue from the infected groups of fish. Aeromonas salmonicida was isolated from 90 to 100% of the fish that died in each temperature group. However, among fish that survived, it was isolated from only 3 of 200 individuals that were examined. Apparently natural defense mechanisms had eliminated the bacterium from the tissues of most of the survivors.

Table 2. Recovery of Aeromonas salmonicida by bacteriological culture of kidney tissue from juvenile chinook salmon.

Water temperature	Proportion of experimentally infected fish yielding positive cultures at autopsy			
	Fatally infected fish		Surviving fish	
	No. positive / No. tested	Percent positive	No. positive / No. tested	Percent positive
72 F	41/42	98	0/1	0
69 F	42/42	100	0/3	0
64 F	37/37	100	0/13	0
59 F	28/28	100	5/22	23
54 F	35/35	100	5/15	33
49 F	23/24	96	4/26	15
44 F	13/13	100	7/37	19
39 F	0/4	0	28/46	61

Aeromonas salmonicida was not recovered from any of twenty percent of the control fish that were cultured.

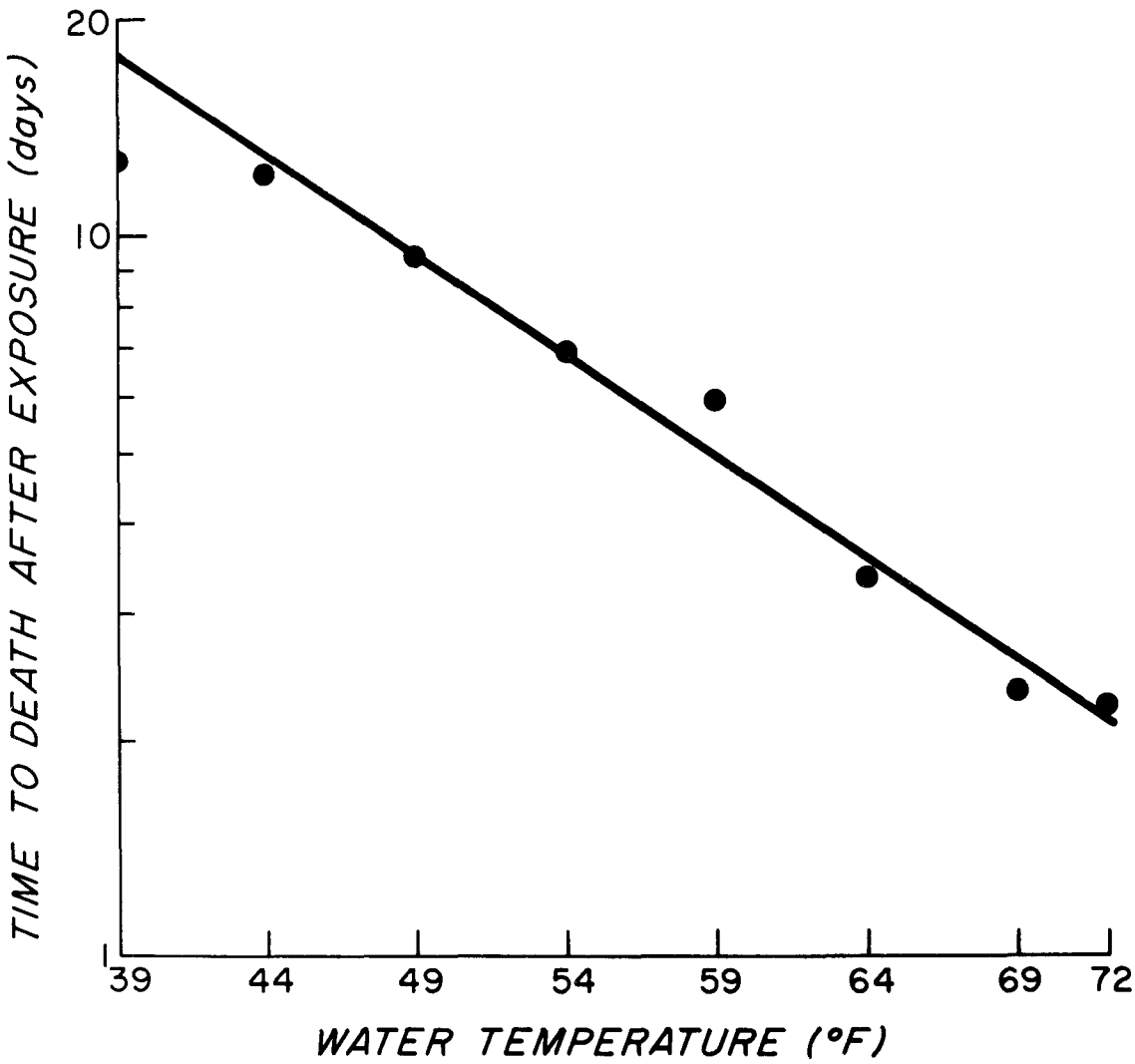

Fig. 1. Relationship between water temperature and the log of time to death after infection of juvenile chinook salmon with Aeromonas salmonicida.

Table 3. Effect[a] of water temperature on Aeromonas salmonicida infection in juvenile steelhead trout.

Water temperature[c]	Fraction of each group that died				Percent mortality; 2 expt. combined		Mean time from infection to death in days
	Experiment 1		Experiment 2				
	Infected[b]	Controls	Infected[b]	Controls	Infected[d]	Controls	
74°F	24/25	24/25	24/25	24/25	96	96	2.6
69°F	24/25	4/25	24/25	1/25	96	10	2.7
64°F	18/25	4/25	24/25	0/25	84	8	5.3
59°F	14/25	0/25	13/25	0/25	54	0	4.9
54°F	9/25	5/25	6/25	1/25	30	12	15.3
49°F	6/25	1/25	8/25	1/25	28	4	13.5
44°F	0/25	0/25	5/25	0/25	10	0	20.3
39°F	1/25	0/25	0/25	0/25	2	0	n.d.[e]

[a]The average weight of the fish at the beginning of the experiment was 35 gm.

[b]Fish were infected by an intraperitoneal injection of 0.1 ml of a 19 hour culture of A. salmonicida strain SS-70 BE-3 in Brain Heart Infusion broth, diluted to contain about 600 colony forming units, or about 2 LD_{50}. Control fish received a sham injection of 0.1 ml of sterile phosphate buffered saline.

[c]All groups of fish were held at the indicated temperatures for 33 days. Dead fish were collected daily, autopsied, and cultures made from kidney tissue. Surviving experimental fish and 5 controls from each temperature group were autopsied and kidney cultures prepared.

[d]The least significant difference between percent mortality values was determined to be 16.22% at the 0.05 probability level (Appendix, page 59).

[e]n.d. indicates no data.

Table 4. Recovery of Aeromonas salmonicida by bacteriological culture of kidney tissue from juvenile steelhead trout.

Water temperature	Proportion of experimentally infected fish yielding positive cultures at autopsy			
	Fatally infected fish		Surviving fish	
	No. positive / No. tested	Percent positive	No. positive / No. tested	Percent positive
74°F	43/48	90	0/2	0
69°F	44/48	92	0/2	0
64°F	39/42	93	0/8	0
59°F	26/27	96	0/23	0
54°F	15/15	100	2/35	6
49°F	13/14	93	0/36	0
44°F	5/5	100	0/45	0
39°F	1/1	100	1/49	2

Cultures were also prepared from twenty percent of the uninfected control fish, but A. salmonicida was not recovered from any of them.

As in the chinook salmon experiments, a linear relationship between the log of the time (number of days) to death and water temperature was observed and confirmed by regression analysis (Fig. 2). The correlation coefficient was -0.7635, a significant value, and R^2 was 0.5829 (Appendix, page 66). The latter figure indicates that about 58% of the variation in time to death was accounted for by temperature. The mean of times to death decreased from 20.3 days at 44 F to 2.7 days at 69 F. Thus the accelerating effect of higher tempertures was again evident.

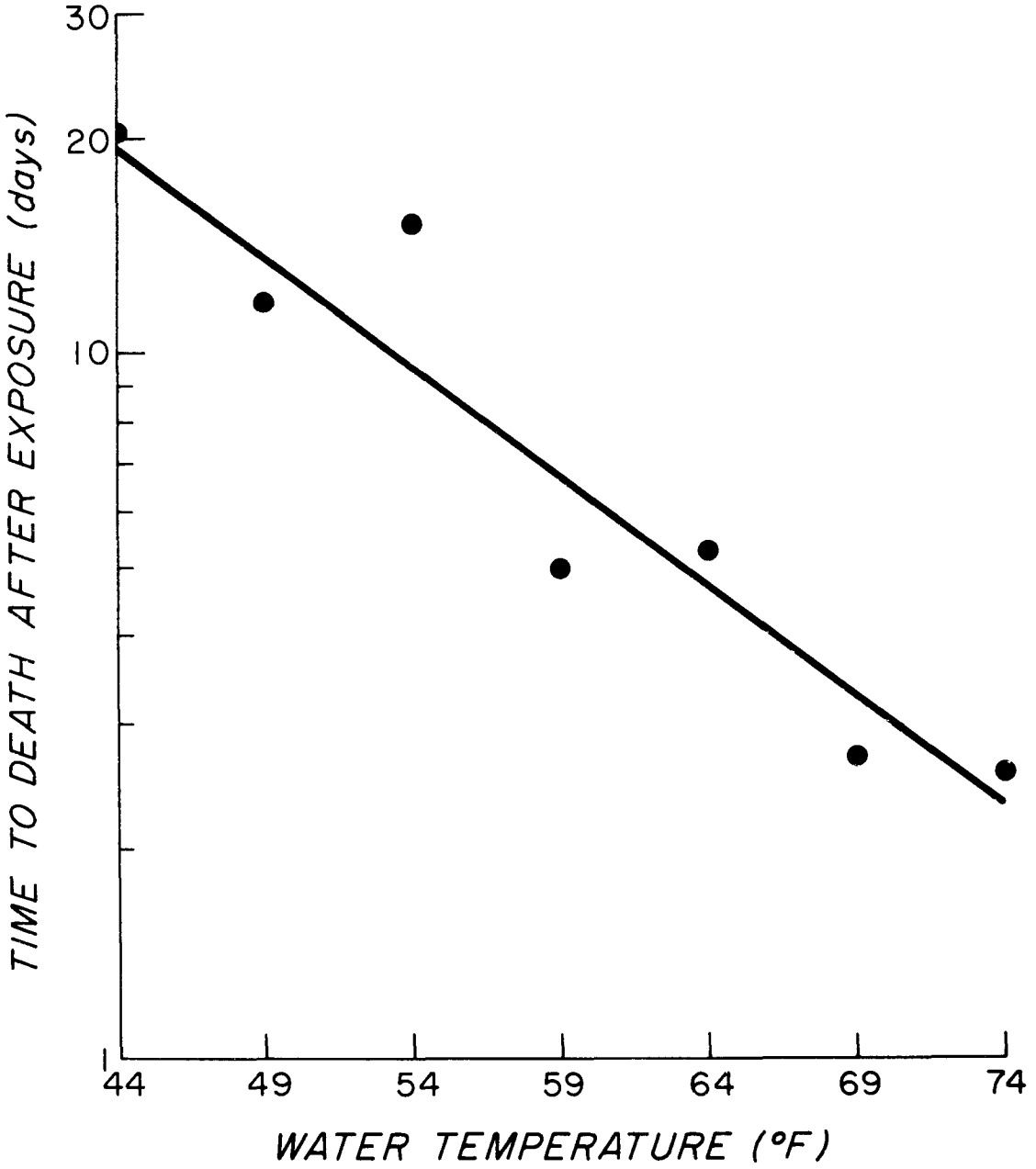

Fig. 2. Relationship between water temperature and log of time to death after infection of juvenile steelhead trout with Aeromonas salmonicida.

Effect of Temperature on Infection with Aeromonas liquefaciens

Experiments providing data concerning the influence of water temperature on experimental infection of juvenile steelhead trout with Aeromonas liquefaciens have been reported previously (1). Similar studies were repeated on juvenile coho and spring chinook salmon and the results are presented below.

Two parallel experiments were conducted concurrently with spring chinook salmon. Four hundred fish, averaging 18 gm in weight, were used in each experiment. They were distributed randomly among 16 tanks, 25 fish per tank. The experimental design was as before except that fish to be infected received an intraperitoneal injection of 0.05 ml of a suspension of A. liquefaciens in PBS containing 1.22×10^7 organisms, or about 2 LD_{50}. The inoculum was prepared from an 18 hour culture of the bacterium in BHI broth by washing and resuspending in PBS. Uninfected control fish received an injection of 0.05 ml of sterile PBS. All groups of fish were at their experimental temperature levels for 12 days post injection. Dead fish were collected daily and cultures of kidney tissue were prepared on BHI agar plates. Identification of A. liquefaciens colonies was made by agglutination with specific antiserum. At termination of the experiments all surviving fish in the infected groups, and five uninfected controls from each temperature group were autopsied and cultures of kidney tissue prepared on BHI agar.

Results of the two experiments are shown in Table 5. The percent mortality, which was 78% at 72 F, decreased progressively with decreasing temperatures to 38% at 54 F, and to less than 15% at 49 F or below. A few deaths occurred among uninfected controls in all temperature groups. Recovery of A. liquefaciens by culture of kidney tissue of infected fish and controls is recorded in Table 6. Ninety four to 100% of fatally infected fish held at temperatures of 54 to 72 F yielded cultures of the organism, while no cultures were recovered from any of the infected fish that survived. Apparently the survivors had been able to eliminate the inoculated bacteria from the tissues during the 12 day experimental period. The organism was not recovered from the 20% of the uninfected control fish that were examined. The mean time to death was very short with this pathogen and was not significantly affected by temperature in the range from 59 to 72 F. Apparently fatal infections were suppressed at temperatures of 49 F and below, and were progressively enhanced as the temperature increased from 54 to 72 F. These results in juvenile chinook salmon are very much like those previously reported, where the same pathogen was used to infect steelhead trout (1).

When infection in juvenile coho salmon with A. liquefaciens was studied, the experimental design was essentially the same as that used with the chinook but certain details were different. Thirty five coho were used in each group instead of 25, and their average weight was 25 grams. Fish

Table 5. Effect of water temperature on Aeromonas liquefaciens infection in juvenile spring chinook salmon.[a]

Water temperature[c]	Fraction of each group that died				Percent mortality; 2 expt. combined		Mean time from infection to death in days
	Experiment 1		Experiment 2				
	Infected[b]	Controls	Infected[b]	Controls	Infected[d]	Controls	
72°F	18/25	2/25	21/25	0/25	78	4	0.9
69°F	16/25	1/25	18/25	0/25	68	2	1.2
64°F	19/25	2/25	12/25	1/25	62	6	1.4
59°F	14/25	0/25	14/25	1/25	56	2	1.3
54°F	7/25	0/25	12/25	1/25	38	2	3.1
49°F	0/25	2/25	0/25	0/25	0	4	-
44°F	3/25	0/25	4/25	6/25	14	12	-
39°F	4/25	5/25	2/25	2/25	12	14	-

[a]The average weight of the fish at the beginning of the experiment was 18 gm.

[b]Fish were infected by an intraperitoneal injection of 0.05 ml of an 18 hour culture of A. liquefaciens strain K-1 in Brain Heart Infusion broth, washed and resuspended in phosphate buffered saline. The injected dose contained about 1.2×10^7 organisms, representing about 2 LD_{50}. Control fish received a sham injection of 0.05 ml of sterile phosphate buffered saline.

[c]All groups of fish were held at the indicated temperatures for 12 days except for the 72 F group which was terminated on day 8. Dead fish were collected daily, autopsied, and cultures made from kidney tissue. After termination, all surviving experimental fish and 5 controls from each temperature group were autopsied and kidney cultures prepared.

[d]The least significant difference between percent mortality values was determined to be 13.3% at the 0.05 probability level (Appendix, page 60).

Table 6. Recovery of *Aeromonas liquefaciens* by culture of kidney tissue of juvenile spring chinook salmon.

Water temperature	Proportion of experimentally infected fish yielding positive cultures at autopsy			
	Fatally infected fish		Surviving fish	
	No. positive / No. tested	Percent positive	No. positive / No. tested	Percent positive
72°F	39/39	100	0/11	0
69°F	33/34	97	0/16	0
64°F	29/31	94	0/19	0
59°F	27/28	97	0/22	0
54°F	19/19	100	0/31	0
49°F	0/0	-	0/50	0
44°F	0/4	0	0/46	0
39°F	0/2	0	0/48	0

Cultures were also prepared from twenty percent of the uninfected fish and none were found to be infected.

to be infected received an intramuscular injection of 0.05 ml of an 18 hour culture of A. liquefaciens, strain JZ-45 in BHI broth, diluted to contain about 2.9×10^7 cells, or about 1.5 LD_{50}. Uninfected controls received the same volume of a sterile filtrate of the broth culture, similarly diluted. All experimental groups were held at their respective temperatures for 15 days. Dead fish were collected daily and cultures of kidney tissue prepared on BHI agar plates. Ten control fish from groups held at 54 F and above were sacrificed and examined by kidney culture at the end of the experiment.

The data obtained in the coho salmon experiments appears in Table 7. All of the inoculated fish in the experimental groups held at 64 F and above succumbed to infection with this pathogen, as did 97% of those held at 59 F. At 54 F, mortality decreased significantly to 41%, and no deaths occurred at 49 F or below. All control fish remained healthy during the experimental period. Results of culturing kidney tissue of fatally infected fish and control fish are given in Table 8. All of the inoculated fish that died in groups held at 54 F and above yielded cultures of A. liquefaciens. This organism was isolated from three specimens among 50 control fish from these five temperature groups that were examined by culture.

The mortality data from the coho salmon resemble closely the comparable data from steelhead trout (1) and chinook salmon. Fatal infections did not develop at water temperatures of 49 F or below, but appeared first at 54 F, affecting about 41% of the fish. At higher temperatures, mortality increased rapidly to 100%. It is also evident from the data in Table 7 that the disease process in the infected fish progressed most rapidly at the higher temperatures. This is indicated by the mean times from infection to death which increased from about 1 day at 74 and 69 F to 4.7 days at 54 F.

As reported in the A. salmonicida experiments, when the log of the number of days from inoculation until death was plotted against water temperature, the relationship was found to be linear in the range from 54 to 69 F (Fig. 3). Regression analysis revealed a correlation coefficient of -0.7017 (Appendix, page 67) which was highly significant, and a coefficient of determination (R^2) of 0.4924. The latter indicates that the time to death was about 49% dependent on temperature.

In view of the fact that percent mortality due to A. liquefaciens in salmonids increased progressively with increasing water temperature and that the disease process was accelerated by higher temperatures, it was of interest to determine the relationship of temperature to the growth rate of the organism in vitro. Accordingly peptone beef extract glucose broth was inoculated with the K-1 strain at an initial concentration of 10^4 to 10^5 cells per ml and distributed in 100 x 13 mm screw cap tubes in three ml aliquots. Groups of these tubes were then incubated at each of

Table 7. Effect of water temperature on Aeromonas liquefaciens infection in juvenile coho salmon.[a]

Water temperature[c]	Fraction of each group that died				Percent mortality; 2 expt. combined		Mean time from infection to death in days
	Experiment 1		Experiment 2				
	Infected[b]	Controls	Infected[b]	Controls	Infected[d]	Controls	
74°F	35/35	0/35	35/35	0/35	100.0	0	1.0
69°F	35/35	2/35	36/36	0/35	100.0	2.9	1.1
64°F	36/36	1/35	34/34	0/35	100.0	1.4	1.5
59°F	33/35	0/35	35/35	0/35	97.2	0	2.3
54°F	16/35	0/35	13/35	0/35	41.4	0	4.7
49°F	2/35	0/35	0/35	0/35	2.9	0	n.d.[e]
44°F	0/35	0/35	0/35	0/35	0	0	---
39°F	0/35	0/35	0/35	0/35	0	0	---

[a]The average weight of the fish at the beginning of the experiment was 18 gm.

[b]Fish were infected by an intramuscular injection of 0.05 ml of an 18 hour culture of A. liquefaciens strain J-45 in BHI broth, diluted to contain about 2.9×10^7 cells, or about 1.5 LD_{50}. Control fish received a sham injection of 0.05 ml of a sterile filtrate of the broth culture, similarly diluted.

[c]All groups of fish were held at the indicated temperatures for 25 days. Dead fish were collected daily, autopsied, and cultures made from kidney tissue. Ten control fish from groups held at 54 F and above were sacrificed and kidney cultures were prepared at the end of the experiment.

[d]The least significant difference between percent mortality values was determined to be 5.02% at the 0.05 probability level. (Appendix, page 61).

[e]n.d. indicates inadequate data.

Table 8. Recovery of Aeromonas liquefaciens by bacteriological culture of kidney tissue from juvenile coho salmon.

Water temperature	Proportion of experimentally infected fish yielding positive cultures at autopsy	
	No. positive / No. tested	Percent positive
74 F	70/70	100
69 F	70/70	100
64 F	70/70	100
59 F	68/68	100
54 F	29/29	100

Ten control fish from each temperature group were also examined by culturing kidney tissue. A. liquefaciens was isolated from 3 of the 50 specimens.

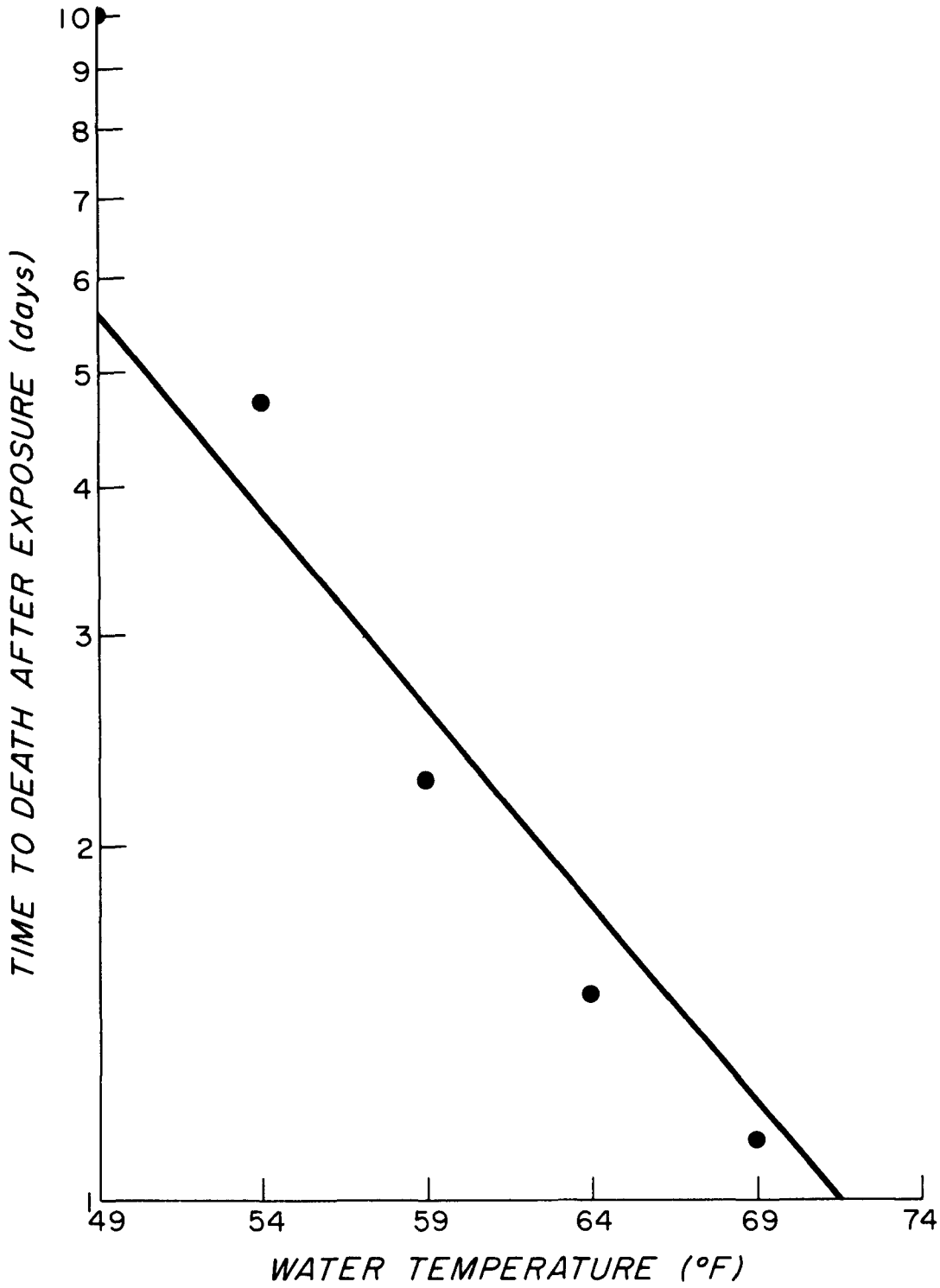

Fig. 3. Relationship between water temperature and log of time to death after infection of juvenile coho salmon with Aeromonas liquefaciens.

the eight temperatures used in experiments with fish. Growth was determined at selected intervals by measuring optical density at 650 nm. The growth rates observed are shown in Fig. 4. At 39 F growth was barely measurable and was quite slow at 44 F. The rate increased significantly at 49 F and was still greater at 54 F. Temperatures from 59 to 74 F resulted in further increases in rates, which reached a maximum at 74 F. When these data are compared with the mortality data in experiments with salmonid fish it is apparent that the temperatures from 49 to 74 F, which gave the highest in vitro growth rates, are also those that produced the highest mortality and the shortest mean times from infection to death in inoculated fish. The three lowest temperatures, 39, 44 and 49 F, with the lowest growth rates, were those which consistently suppressed the development of fatal infections in fish. Thus it seems reasonable to assume from these in vitro data that the effect of temperature on the growth rate of the pathogen is in part responsible for the effects of temperature on the disease process in fish.

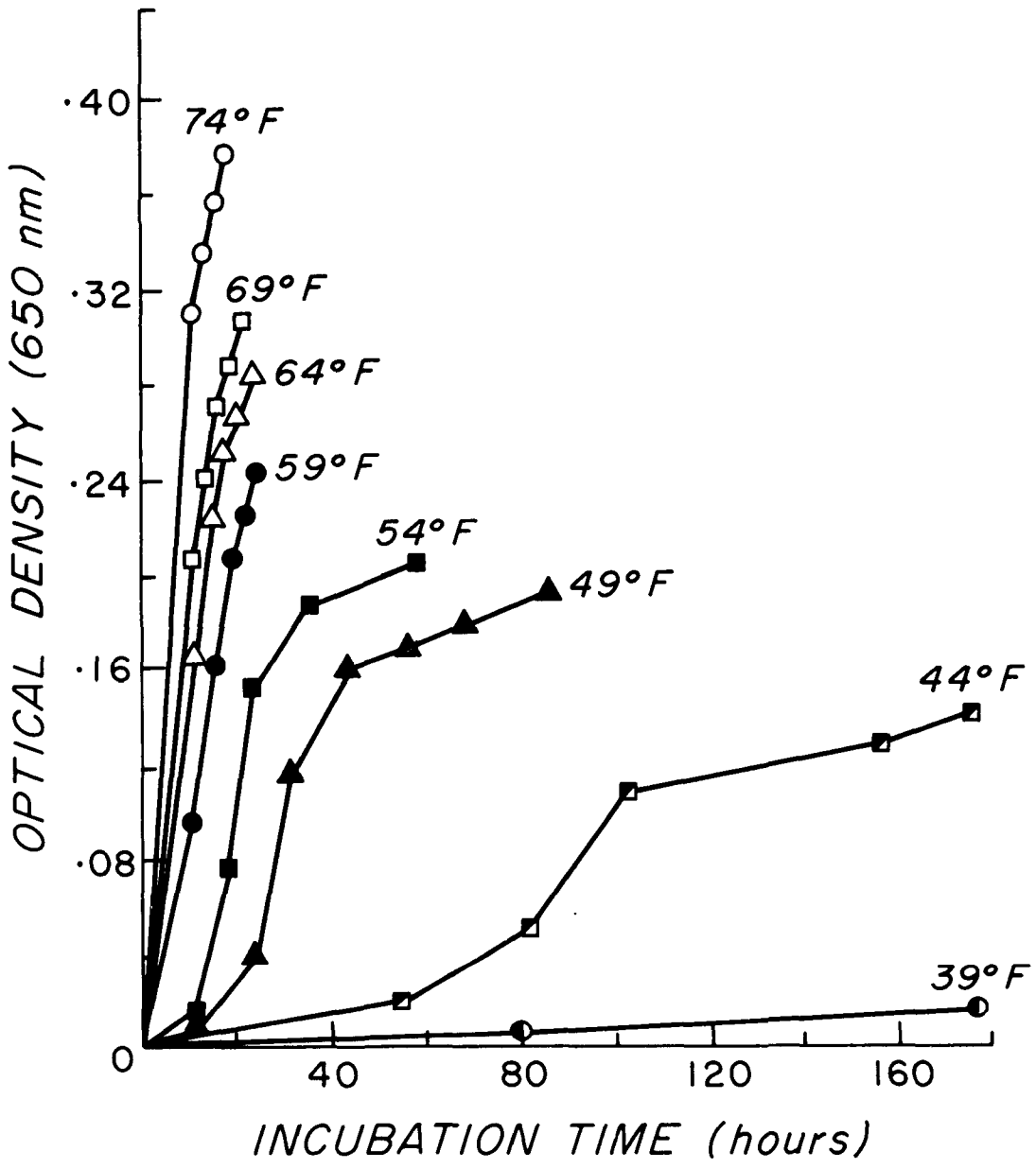

Fig. 4. Effect of temperature on growth rate of Aeromonas liquefaciens in peptone beef extract glucose broth.

Discussion

The effect of water temperature on infection with A. salmonicida and A. liquefaciens was studied in coho and spring chinook salmon, and steelhead trout. Temperature effects on A. salmonicida infections were very similar in all three salmonid species. Fatal infection was suppressed by temperatures of 39 or 44 F, and was progressively enhanced as temperatures increased from 49 to 69 F. This was indicated both by the mortality rates and by the mean times to death, which were longest at the low temperatures, but decreased progressively as temperatures increased. As reported previously (1) these effects appear to be related to comparable effects on growth of the organism in vitro. Such growth was barely detectable at 39 F, markedly retarded at 44 F, and increased progressively in rate as the temperature increased from 49 to 69 F. From the mortality data presented it might be anticipated that natural outbreaks of furunculosis among juvenile salmonids would be most likely when water temperatures are 50 F or higher.

In the case of the A. liquefaciens infections, the effects of temperature were similar among the three salmonid species. However, in these experiments the three lowest temperatures (39, 44 and 49 F) essentially eliminated any fatal infections in coho salmon and steelhead trout during the experimental period (1). Thus, suppression was somewhat more complete at these temperatures than in the A. salmonicida infections. Mortality increased progressively as water temperature increased from 54 to 72 or 74 F. The effects of temperature on the A. liquefaciens infections also, appear to be related to the growth rates in vitro at the various experimental temperatures. Growth was negligible at 39, very slow at 44 F, and increased in rate with temperature increments of five degrees from 49 to 74 F. The experimental data on mortality seem to indicate that fatal disease due to A. liquefaciens probably does not occur in juveniles of the three salmonid species studied until water temperature rises above 49 F.

SECTION VI

EFFECTS OF WATER TEMPERATURE ON INFECTION OF STEELHEAD TROUT BY *FLEXIBACTER COLUMNARIS*

Materials and Methods

In an earlier report (1) experiments were described in which the effects of water temperature on infection by *Flexibacter columnaris* were investigated in coho salmon, spring chinook salmon and rainbow trout. These studies have now been expanded to include juvenile steelhead trout, which averaged 13 grams in weight. These are the same species as rainbow trout (*Salmo gairdneri*), but differ by being anadromous.

The experimental design was essentially the same as previously described in Section V for experiments with *A. salmonicida*.

The *F. columnaris* isolate used in these experiments was obtained from a lesion on the gill of an adult spring chinook salmon at the Fish Commission of Oregon, Dexter Dam Holding Pond, Willamette River. It was passed in coho salmon fry seven times to increase its virulence. After the seventh passage the culture was lyophilized. Immediately prior to each experiment a lyophilized specimen was cultivated in Cytophaga broth (2) and passed once in juvenile steelhead trout. Several isolates from this final fish passage were collected and pooled to prepare the inoculum for infecting fish in the temperature experiments. Each isolate was grown in tryptone yeast infusion broth (3) for about 20 hours at 24 C. The resulting cultures were then mixed for the preparation of the inoculum. The optical density of the latter was adjusted to 0.1 at 525 nm. Infection of the fish in the experimental aquaria was then accomplished by addition of the mixed culture to the aquarium water. The water supply was cut off for a 10 minute period and the volume (water plus fish) in the tank reduced to about 20 liters. A sufficient volume of the culture was then added to give a 1:20 dilution, which represented 3 to 6 x 10^6 colony forming units per ml. After the 10 minute exposure period, the flow of water was resumed.

In control groups of fish which were not exposed to the pathogen, the volume of water in each tank was reduced to the level in exposed tanks, and water flow was interrupted and resumed in the same manner. Dead fish were collected daily during the 25 day period following infection, and cultures were prepared by streaking gill or kidney tissue on Cytophaga agar.

Experimental Phase

The results of the two experiments are presented in Table 9. All of the exposed fish held at 69 F and 92% of those held at 64 F succumbed within the 25 day observation period. Mortality dropped significantly to 56% in the groups held at 59 F, and was only 16% among those held at 54 F. No deaths occurred at 49 or 39 F, and only one at 44 F. Flexibacter columnaris was isolated from 96 to 100% of exposed fish that died at 54 to 69 F by culturing gill and kidney tissue.

These experiments clearly demonstrated the effect of temperature on the incidence of fatal infection in juvenile steelhead trout exposed to this pathogen. Mortality increased progressively with increasing temperature in the range from 54 to 69 F. The disease was effectively suppressed at 49 F and below. The 74 F water temperature was not tolerated by these fish, as all of the unexposed control fish died during the experimental period. Flexibacter columnaris was not recovered from these animals at autopsy, and these are presumed to be thermal deaths.

A second effect of water temperature on this infection became apparent when the mean number of days from exposure to death was plotted on a log scale against temperature. The relationship between these two variables was found to be linear, as shown in Fig. 5. A correlation coefficient of -0.7604 was calculated and found to be highly significant. The coefficient of determination (R^2) was shown to be 0.5783, indicating that about 58% of the variation in the log of the time to death was accounted for by water temperature. The equation and the data used in computing it are shown in Appendix, page 68. The mean time to death decreased from 7.6 days at 54 F to 1.7 days at 69 F. Thus the progress of fatal infection was accelerated at the higher temperatures and retarded at the lower temperatures.

Although precise measurements of growth of this strain of F. columnaris in vitro were difficult because of the strong tendency of the cells to form clumps in liquid medium, growth rates were determined approximately at 49 to 74 F by measuring the optical density of static cultures in tryptone yeast infusion broth. In this temperature range growth was most rapid at 64 to 74 F. At 59 F there was a lag period of about 47 hours before growth began, and the growth rate was less than at 64 F. At 54 and 49 F no significant growth was observed, even after 100 hours incubation. Thus the optimal temperature range for growth in vitro was correlated with the temperature that produced the highest mortality and the shortest mean times to death. Furthermore, the 49 and 54 F temperatures, which were very unfavorable for growth of the organism in vitro were associated with little or no mortality in the groups of exposed fish.

Table 9. Effect of water temperature on mortality of juvenile steelhead trout infected with *Flexibacter columnaris*.[a,b]

Water temperautre	Fraction of each group that died[d]				Percent mortality; 2 expt. combined		Percent of deaths infected with F. columnaris[d]	
	Experiment 1		Experiment 2					
	Infected	Controls	Infected	Controls	Infected[c]	Controls	Infected	Controls
74 F	25/25	25/25[e]	25/25	25/25[e]	100	100	100	0
69 F	25/25	1/25	25/25	0/25	100	2	100	0
64 F	22/25	2/25	24/25	3/25	92	10	98	0
59 F	13/25	0/25	15/25	0/24[f]	56	0	96	0
54 F	2/25	0/25	6/25	2/25	16	4	100	0
49 F	0/25	0/25	0/25	0/24[f]	0	0	0	0
44 F	0/25	0/25	1/25	0/25	2	0	0	0
39 F	0/25	1/25	0/25	0/24[f]	0	2	0	0

[a]Average weight of fish when experiment started was 13 g.

[b]Experiment was terminated 25 days after exposure to *F. columnaris*.

[c]The least significant difference between values for percent mortality from combined experiments was determined by analysis of variance to be 8.2% at the 0.05 probability level (Appendix, page 62).

[d]Based on recovery of the organism by culture at autopsy.

[e]All control fish at 74 F died during the first 13 days of the holding period.

[f]One fish unaccounted for.

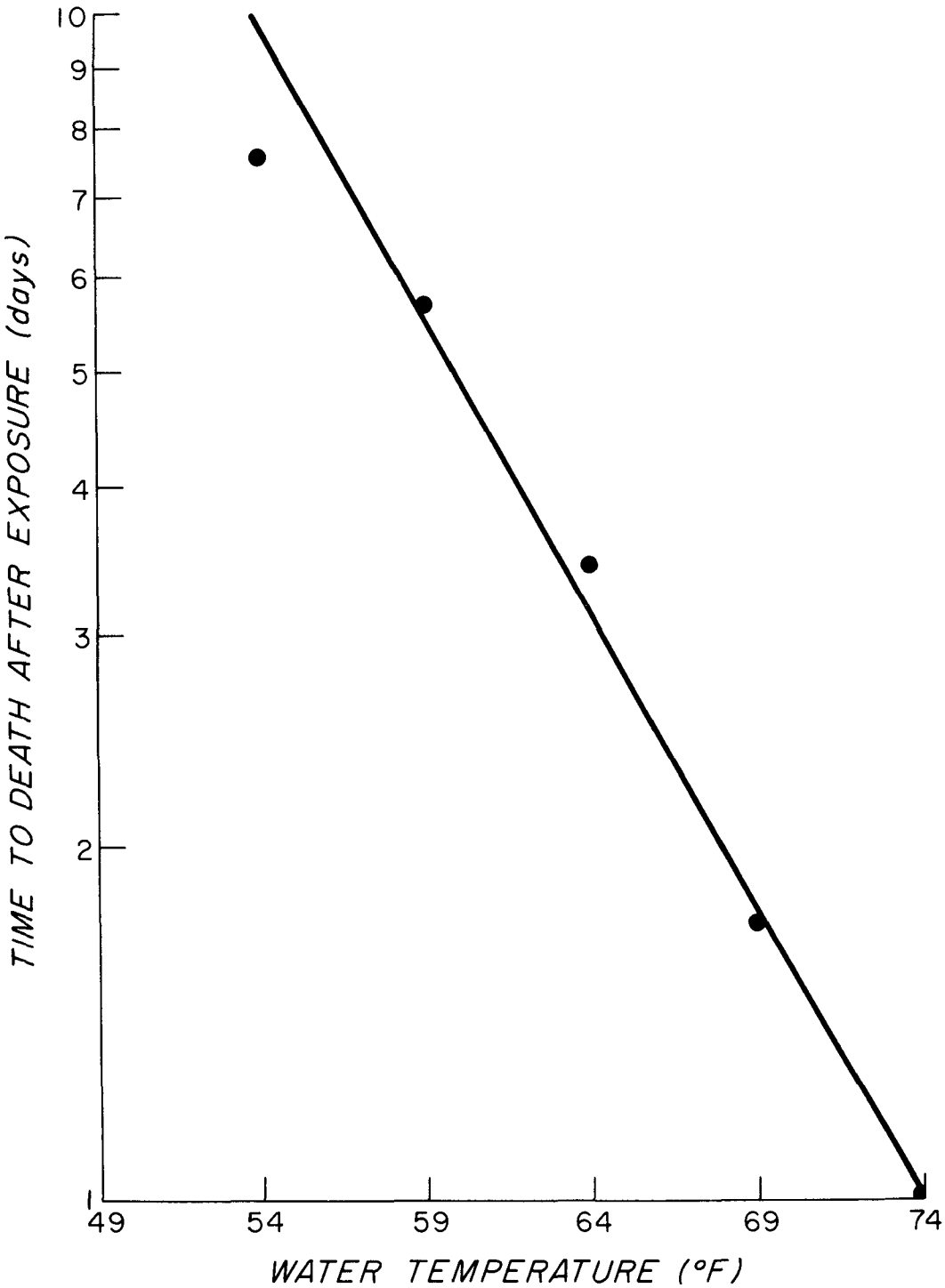

Fig. 5. Relationship between water temperature and log of time to death after exposure of juvenile steelhead trout to Flexibacter columnaris.

Discussion

The results indicate that the effects of water temperature on experimental infection of steelhead trout with F. columnaris were very similar to those in coho and spring chinook salmon (1). In all three salmonid species, the lowest temperatue at which deaths were observed due to infection by F. columnaris was 54 F, while mortality increased progressively with increasing temperature in the range from 54 to 69 F. At 49 F and below, conditions were unfavorable for progress of the infection. The effect of temperature on defense mechanisms of the host is not known. However, the rapid growth of F. columnaris in vitro at temperatures of 64 to 74 F, and the very limited growth at 54 F and below suggest that the effect of temperature on mortality is due in part to its effect on growth of the organism in the host's tissues.

The determination of the mean time between exposure to the pathogen and death of the fish provides a measure of the rate at which fatal infection develops. In all three salmonid species, this data showed clearly that progress of the infection was accelerated progressively as the temperature increased from 54 to 69 or 74 F. At 49 F and below no fatalities attributable to F. columnaris occurred in any of the three species. It seems likely that this accelerating effect of increasing temperature may also be due in some degree to stimulation of growth of the bacterium.

The experiments reported were conducted under controlled conditions in an experimental fish disease laboratory and the significance of the results with respect to the effects of temperature on the occurrence of columnaris disease under natural conditions deserves consideration. It was important to use water containing no fish pathogens in the experimental aquaria, rather than a natural river water, in order to minimize the occurrence of disease symptoms and mortality caused by pathogens other than the one being studied.

Although it cannot be inferred that the results obtained define exactly the relationship of temperature to mortality from columnaris disease that could be expected under natural conditions, they are not in conflict with reported studies of this relationship as it affects the natural disease. The influence of higher temperatures in streams and hatchery troughs on incidence of the disease and initiation of the infection has been reported by other workers (4, 3, 5, 6).

Ordal and Pacha (4) found that under experimental conditions, highly virulent strains of F. columnaris could initiate infection in fish at water temperatures as low as 55 F, while strains of low virulence could do so only when the temperature was increased to 68 F. The strain used in the experiments described here appears to fall in their category of intermediate virulence, as it killed 100% of experimental fish exposed by water contact at 69 F within 96 hours but not within 48 hours.

The data presented define the effects of temperature on columnaris infection without confusion from the activity of other pathogens in the water, and therefore with somewhat greater precision than reported by other workers. These results do provide additional guidelines for future understanding and efforts to control this disease. Considering the available evidence from all investigators, and the existence of strains of F. columnaris of high and low virulence, it appears that water temperatures below 54 F are required for complete suppression of the disease in salmonid populations that have been exposed to the pathogen.

SECTION VII

EFFECT OF WATER TEMPERATURE ON BACTERIAL KIDNEY DISEASE IN SALMONIDS

Materials and Methods

The influence of water temperature on experimental infection with bacterial kidney disease was studied in juvenile coho salmon and steelhead trout. Two strains of the bacterium (Corynebacterium sp.) used were isolated from spring chinook and coho salmon at the Hood River and Nehalem Hatcheries, respectively. Stock cultures were maintained on Cysteine blood agar (7) modified by using calf serum in place of whole blood.

Inoculum for infecting fish was prepared as a suspension of the organism in physiological saline. In experiments with coho salmon the suspension contained about 2.5×10^8 bacteria in 0.05 ml, and in steelhead trout experiments the concentration was about 2.9×10^8 in this volume.

Juvenile coho salmon averaged 6.5 grams in weight, while steelhead trout averaged 18 grams. Fish in groups to be infected were injected intraperitoneally with 0.05 ml of the bacterial suspension, and control fish received the same volume of sterile physiological saline.

The experimental design was almost the same as previously described in Section I for A. salmonicida. Fourteen aquaria, each containing 25 fish were used in each experiment. Seven streams of tempered water, varying from 39 to 69 F in five degree increments were provided, and two aquaria were maintained at each temperature. Seven of them, one at each temperature, were assigned to groups of fish to be infected, while the remaining seven were used for uninfected controls. The fish were tempered to the various temperature levels by the method described in Section V. Two complete and identical experiments were conducted concurrently, each one consisting of seven infected groups and seven controls.

Dead fish were collected daily during the experimental period, autopsied, and smears of kidney tissue prepared on glass slides. These were gram stained and examined microscopically for the presence of the typical kidney disease bacteria. This criterion was used to establish kidney disease as the cause of death. Bacterial cultivation of the organism was considered impractical in this case because of its slow growth rate and fastidious character.

Experimental Phase

The results of duplicate experiments with kidney disease in juvenile coho salmon are presented in Table 10. The inoculated and control groups of fish were observed at their respective temperature levels over a period of 112 days. At this time deaths among experimental fish had ceased to occur in all groups except those at 39°F. The percent mortality values in Table 10 were based on dead fish in which the specific pathogen was demonstrated at autopsy. This was done because the lengthy period during which these fish were held provided greater opportunity for occurrence of deaths from causes other than kidney disease. Such deaths are especially evident among the control groups of steelhead in Table 11.

In Table 10 the combined data from the two experiments with coho show that mortality approached 100% in the temperature range of 44 to 54 F. As the temperature increased above 54 F, mortality declined progressively, and was only 40.8% at 64 F and 13.6% at 69 F. When the experiment was terminated after 112 days, mortality at 39 F had reached 63.4%, but fish were still dying from kidney disease. At this time the 15 surviving fish in the infected groups, and the 46 controls were tempered to 54 F water by the method described in Section V. Within two weeks all of the fish in the infected groups had succumbed to kidney disease, whereas none of the control fish had died. This indicates that the pathogen was still viable in the inoculated fish held at 39 F, and that progress of the infection was accelerated by increasing the temperature to 54 F. Presumably if these fish had been allowed to remain at 39 F for a longer period the mortality from kidney disease would have approached 100%.

The relationship between water temperature and log of time to death was linear. This parallels the relationship between these two variables described previously (Section V and VI) for A. salmonicida and F. columnaris infections. The regression analysis of the data is shown in Fig. 6 (and Appendix, page 69). The correlation coefficient was -0.7496, a statistically significant value, and R^2 was 0.5619, indicating that time to death was about 56% dependent on temperature. The values for mean time to death decreased from 84.2 days at 39 F to 22.5 days at 69 F. Increasing temperature was associated with shorter survival and higher mortality.

Although 69 and 64 F were the most unfavorable temperatures for the production of fatal disease, as shown by the mortality data, fish that did develop fatal infection at these temperatures exhibited the shortest survival time between inoculation and death, i.e. 25 and 26 days, respectively. In other words the disease process progressed more rapidly and completely in these fish than in others held at 44 to 59 F. The reason for this is not clear, but it seems possible that the higher temperatures of 69 and 64 F could have exerted a selective influence favoring the growth

Table 10. Effect of water temperature on bacterial kidney disease in juvenile coho salmon.[a]

Water temperature	Fraction of each group that died				Percent mortality;[d] 2 expts. combined		Fraction of deaths[c] due to kidney disease	
	Experiment 1		Experiment 2					
	Infected[b]	Controls	Infected[b]	Controls	Infected[e]	Controls	Infected	Controls
69 F	5/21	1/20	5/23	2/23	13.6	0	6/10	0/3
64 F	10/22	0/23	12/22	2/22	40.8	0	18/22	0/2
59 F	23/25	0/25	15/25	0/25	76.0	0	38/38	0/0
54 F	21/21	0/25	21/21	0/25	100.0	0	42/42	0/0
49 F	25/25	3/23	22/23	0/25	98.0	2.0[f]	47/47	1/3
44 F	21/21	0/25	20/23	1/25	93.2	2.0[f]	41/41	1/1
39 F	11/22	0/23	15/19	1/24	63.4	0	26/26	0/1

[a]The average weight of the fish at the beginning of the experiment was 6.5 gm.

[b]Fish in the inoculated groups received a single intraperitoneal injection of about 2.5×10^8 kidney disease bacteria in 0.05 ml of physiological saline. Fish in control groups received a similar injection of physiological saline.

[c]All groups of fish were held at the indicated temperatures for 112 days. Dead fish were collected daily, autopsied, and smears made from kidney tissue. These were gram stained and examined microscopically for the presence of the pathogen.

[d]Only dead fish in which the specific pathogen was demonstrated at autopsy were considered in calculating percent mortality.

[e]The least significant difference between percent mortality values was determined to be 20.18% at the 0.05 probability level. (Appendix, page 63).

[f]Two percent of the control fish at 44 and 49 F were found to be infected with the causative agent of bacterial kidney disease. The source of these infections is unknown.

Table 11. Effect of water temperature on bacterial kidney disease in juvenile steelhead trout.[a]

Water temperature[c]	Fraction of each group that died				Percent mortality;[d] 2 expts. combined		Fraction of deaths[d] due to kidney disease	
	Experiment 1		Experiment 2					
	Infected[b]	Controls	Infected[b]	Controls	Infected[e]	Controls	Infected	Controls
69 F	20/24	25/26	22/24	3/23	8.3	0	4/42	0/28
64 F	25/27	6/26	26/27	7/23	42.6	0	23/51	0/13
59 F	17/26	3/27	20/27	1/26	49.0	0	26/37	0/4
54 F	23/26	5/27	25/28	1/27	77.7	0	42/48	0/6
49 F	25/25	6/26	27/27	2/27	90.0	0	47/52	0/8
44 F	26/26	1/26	25/25	0/25	98.0	0	50/51	0/1
39 F	4/19	13/25	14/25	5/25	36.4	0	16/18	0/18

[a]The average weight of the fish at the beginning of the exerpiment was 18 gm.

[b]Fish in the inoculated groups received a single intraperitoneal injection of about 2.9×10^8 kidney disease bacteria in 0.05 ml of physiological saline. Fish in control groups received a similar injection of physiological saline.

[c]All groups of fish were held at the indicated temperatures for 112 days. Dead fish were collected daily, autopsied, and smears made from kidney tissue. These were stained by the Gram method and examined microscopically for the presence of the pathogen.

[d]Only dead fish in which the specific pathogen was demonstrated at autopsy were considered in calculating percent mortality.

[e]The least significant difference between percent mortality values was determined to be 15.33% at the 0.05 probability level (Appendix, page 64).

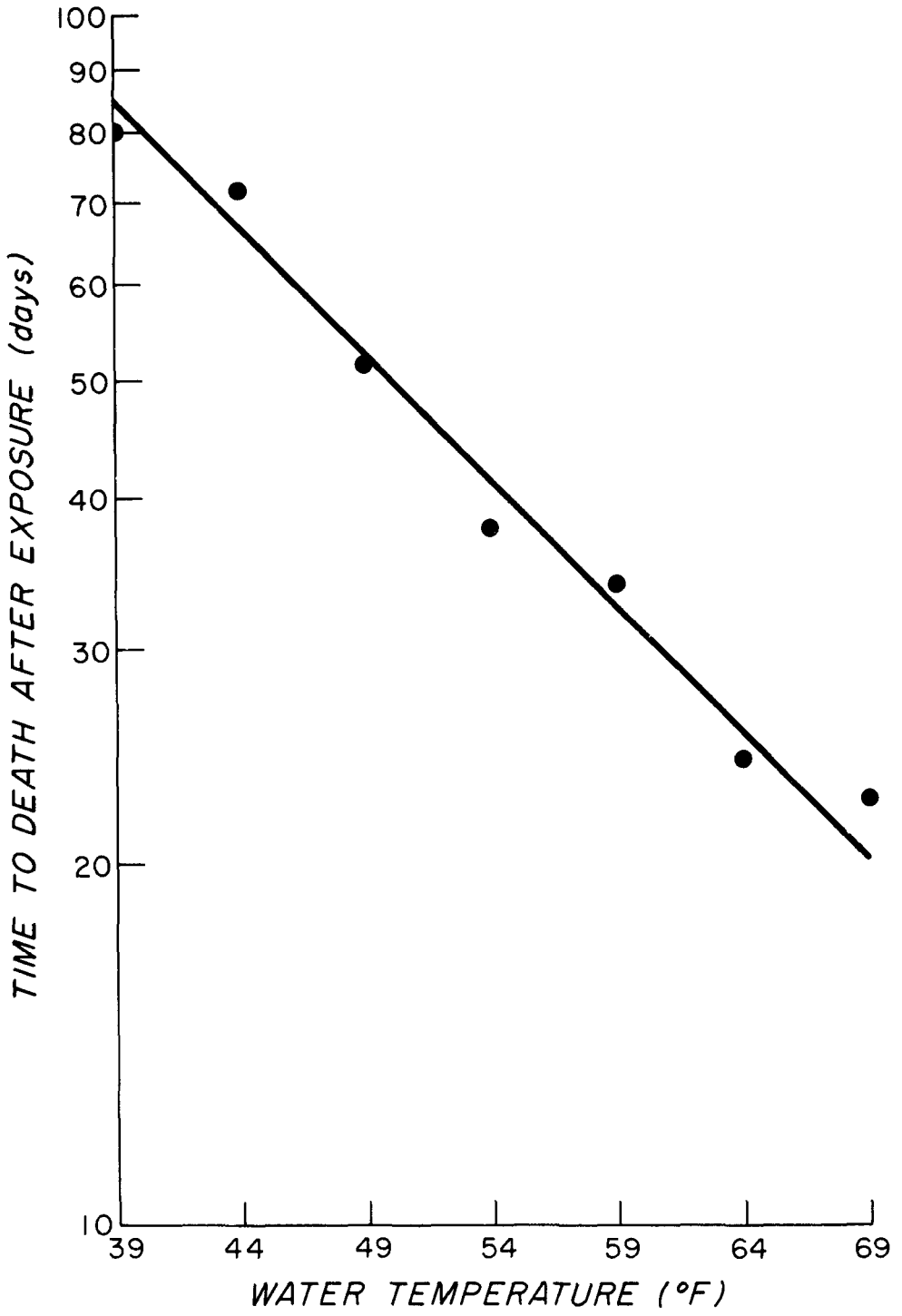

Fig. 6. Relationship between water temperature and log of time to death after infection of juvenile coho salmon with kidney disease bacteria.

of a few mutant bacterial cells in the inoculum, which may have had a greater virulence than the predominant cell population. The mean number of days from inoculation to death increased to 39 at 54 F, 53 at 49 F and 73 at 44 F. At 39 F it was greater than 83 days.

Two parallel experiments with kidney disease were also carried out in juvenile steelhead trout. The experimental design and procedures were the same as in the coho salmon experiments. The influence of water temperature on mortality from this disease is evident from the data in Table 11. Again the percent mortality was greatest in the range of 44 to 54 F, varying from about 78 to 98%. It dropped to 49% at 59 F, about 43% at 64 F, and about 8% at 69 F. At 39 F, about 36% of the inoculated fish had died from kidney disease when the experiment was terminated. However, of the 26 survivors, 21 were found by microscopic examination to be harboring the kidney disease bacterium. Presumably then, as in the coho salmon experiment, if these fish had been allowed to remain at 39 F for a longer period, mortality from kidney disease would have approached 100%.

Thus the data from juvenile steelhead also indicated that the range of 44 to 54 F was optimal for the development of fatal infection by this pathogen, and that higher temperatures had some suppressing effect, which was greatest at 69 F. A complicating factor in these experiments was the prevalence of tail rot in the population of steelhead. It is presumed to be the cause of the deaths among uninoculated control groups shown in Table 11. However, as mentioned previously, in calculating percent mortality values due to kidney disease, only those dead fish were counted which had the specific pathogen. Thus the possible distortion of the data by non-specific infection should be largely eliminated.

The mean times from inoculation of the bacteria until death of the host at the various water temperatures are shown in Fig. 7. Although percent mortality was greatest in the temperature range of 44 to 54 F, the shortest mean time to death was observed at 59 F. Some animals were apparently able to overcome the inoculated dose of the pathogen when the body temperature was held at 59 F, but others in the group were not, and in these animals the disease process progressed more rapidly than in fish maintained at lower temperatures. As the data show, there was a progressive increase in the mean time to death as the temperature decreased from 59 to 39 F. At 64 F the interval was slightly greater than at 59. However at this temperature the efficiency of detection of the pathogen in kidney tissue was less than at lower temperatures, especially in fish that died within two weeks after inoculation. At 69 F the number of deaths was too small to permit a reliable estimation of the mean time to death.

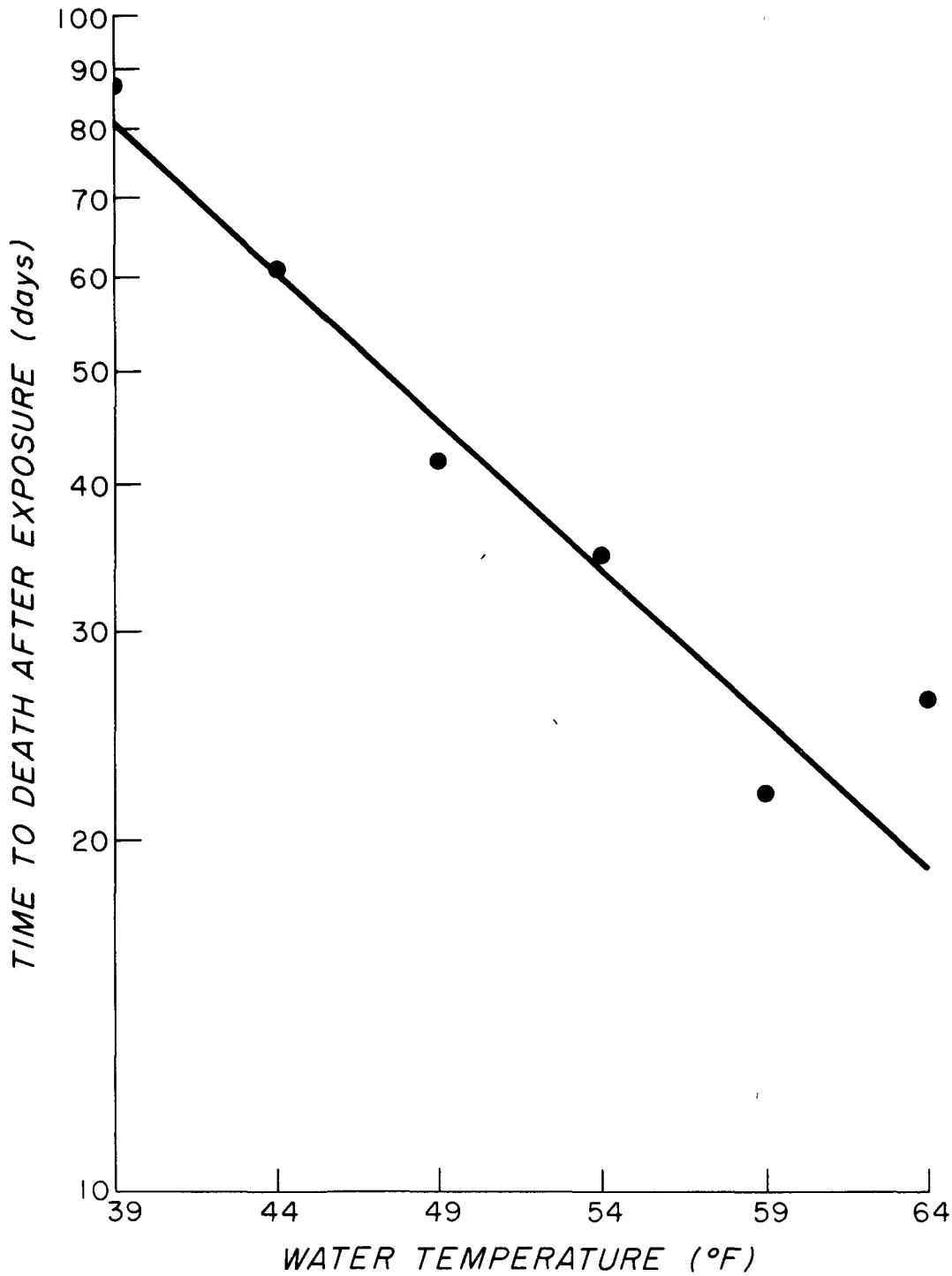

Fig. 7. Relationship between water temperature and log of time to death after infection of juvenile steelhead trout with kidney disease bacteria.

Regression analysis of the data in Fig. 7 again confirmed the linear nature of the relationship between temperature and the mean time to death (Appendix, page 70). The correlation coefficient was -0.7927 and R^2 was 0.6284, showing that about 63% of the variation in time to death was accounted for by the regression line.

Discussion

The experiments described have indicated some definite effects of water temperature on infection of juvenile coho salmon and steelhead trout with the bacterium of kidney disease. In general, these effects are very similar in the two salmonid species. In both cases the temperature range of 44 to 54 F was found to be optimal for the development of fatal infections. At higher temperatures, percent mortality declined progressively, and at 69 F was reduced to values of only 8 to 13%. The above optimal temperature range seems consistent with the observation that mortality from the disease in nature is most apparent in the fall of the year when water temperatures have cooled well below those of summer.

Experimental kidney disease was observed to be a slowly progressing infection in both species. The mean interval from inoculation until death varied from a minimum of about 23 days to more than 89 days, depending on water temperature. In both coho and steelhead the interval increased progressively as the temperature decreased from 59 to 39 F.

Kidney disease is the only one of the bacterial infections of salmonids that have been studied in which temperatures of 64 and 69 F exerted a suppressive effect on the disease process. In the A. salmonicida, A. liquefaciens, and F. columnaris infections higher temperatures were associated with the maximum mortality percentages. It seems quite possible that the much lower optimum range for the kidney disease process may reflect the optimum temperature range for the growth of the bacterium. This is not known precisely, but has been reported to be about 59 F (15 C) (7).

SECTION VIII

EFFECT OF WATER TEMPERATURE ON ANTIBODY FORMATION IN SALMONIDS

Materials and Methods

Juvenile coho salmon averaging 34 grams in weight were tempered to experimental temperatures of 39, 44, 49, 54, 59, 64, 69 and 74 F by the method described in Section V of this report. Each temperature group consisted of 100 fish, which were divided equally between controls and those to be injected with a killed suspension of A. salmonicida. Fifty fish at each temperature received an intraperitoneal injection of 0.1 ml of an emulsion containing equal parts of a BHI broth culture of the organism, killed by 0.3% formalin, and Freund's complete adjuvant. The injected dose represented about 1.5×10^9 cells. Equal numbers of control fish were injected with the same volume of an emulsion containing equal parts of 0.85% saline and the adjuvant. At 15 day intervals after the antigen injection five vaccinated and five control fish at each temperature were bled and individual antibody titers were determined on each serum sample by tube agglutination. The experimental groups held at 74 F had to be eliminated from the experiment because of high mortality rates in both control and vaccinated groups.

An experiment similar to the above was performed, in which groups of juvenile coho salmon, held at the same eight water temperatures, received a single intraperitoneal injection of formalin killed kidney disease bacteria, emulsified in Freund's complete adjuvant. The organisms were grown on cysteine serum agar for two to three weeks at $15^{\circ}C$ and killed by the addition of 0.3% formalin. The emulsion was prepared with equal parts of the killed culture and the adjuvant. The injected dose contained about 2×10^9 cells. Control fish received a similar injection of an emulsion containing 0.85% saline with the adjuvant. At intervals of 15 days after injection, five vaccinated fish and five controls from each temperature group were bled, and the serum specimens titrated for antibody by tube agglutination.

Experimental Phase

Antibody Response to Killed Aeromonas Salmonicida at Various Water Temperatures

The levels of agglutinating antibody produced in juvenile coho salmon held at various water temperatures following a single injection of killed A. salmonicida cells in Freund's adjuvant are shown in Table 12 and Fig. 8. No significant antibody response was found in fish held at 39 F during the 60 day observation period. At 49 F a response was first detected after 30 days, and the level was as high or higher after 60 days. At 54 F the first increase was noted after 30 days, and after 45 days more antibody was present than was found in the fish held at 49 F. Temperatures of 59 and 69 F appeared to be optimal for antibody production, and the highest concentrations were found in fish held at these temperatures 45 days after the injection of antigen. In general these results seem to indicate a progressive enhancement of antibody production as the temperature increases from 39 to 59 F.

In the original design of this experiment it was planned to obtain some information on the comparative degrees of protection produced in the vaccinated fish held at the various experimental temperatures. Twenty five fish from each temperature group including controls were to be challenged by the injection of about 2 LD_{50} of virulent A. salmonicida cells. Before the challenge, it was necessary to temper all of the experimental fish groups held below 59 F up to this temperature, so that the effect of water temperature on the percent mortality after challenge would be eliminated. The tempering process was completed on all groups by the 84th day after the original antigen injection. Twenty five fish from each group were then injected with a dose of A. salmonicida cells calculated to represent about 2 LD_{50}. This portion of the experiment was unsuccessful however, as less than 10% of the unvaccinated control fish developed fatal infection. Hence no information was obtained concerning the effect of temperature on protective antibody, which is not necessarily identical to agglutinating antibody.

On the 84th day after the antigen injection, when the tempering process had been completed, blood specimens were taken from five fish in each vaccinated and control group and agglutination titers determined. At this time only two of the vaccinated groups showed higher mean antibody titers than were found at the 60 day period. The group held initially at 39 F had a titer of 1:512 compared to 1:45 at 60 days. Apparently the gradual elevation of temperature to 59 F had enhanced antibody synthesis. In the groups held at 59 F throughout the entire 84 day period, the titer at 84 days was 1:24,834 compared to 1:2047 at 60 days. No special reason for this increase is apparent, except that this temperature was in the optimal range for antibody formation. In the other temperature groups, the titers at 84 days were not significantly different from those at 60 days, with the exception of those originally held at 69 F. In this case the titer dropped from 1:2,427 at 60 days to 1:323 at 84 days.

Table 12. Agglutinating antibody levels in juvenile coho salmon injected with formalin killed Aeromonas salmonicida in Freund's adjuvant and held at various water temperatures from 39 to 69 F.

Water Temperature	Post injection period in days	Reciprocals of agglutination titers			
		Vaccinated		Controls	
		Mean titer[a]	Titer range	Mean titer[a]	Titer range
54 F	0			30[b]	16-64
39 F	15	11	8-16	11	8-16
	30	56	32-64	24	8-64
	45	42	16-64	23	16-32
	60	45	32-64	42	32-64
44 F	15	28	16-64	32	16-64
	30	84	64-128	64	32-128
	45	85	32-256	24	16-64
	60	194	128-1024	74	32-128
49 F	15	14	8-16	--[c]	--[c]
	30	147	64-512	--	--
	45	74	32-128	--	--
	60	512	32-2048	--	--
54 F	15	24	16-32	24	16-32
	30	295	64-2048	56	32-128
	45	891	128-16384	56	16-128
	60	1787	256-8192	49	16-64
59 F	15	74	16-256	32	32
	30	1349	128-16384	90	64-128
	45	7112	4096-16384	74	64-128
	60	2047	1024-16384	84	64-128
64 F	15	224	32-1024	12	8-16
	30	1728	128-16384	56	32-64
	45	724	256-8192	74	64-128
	60	1778	128-16384	49	32-64
69 F	15	n.d.[d]	n.d.	11	8-16
	30	512	256-1024	n.d.	n.d.
	45	4074	1024-16384	n.d.	n.d.
	60	2427	512-8192	n.d.	n.d.

[a] Values shown are geometric means.
[b] This group of controls received no injections. Other control groups were injected with saline plus Freund's adjuvant.
[c] Insufficient tank space was available for controls at 49 F. For significance at P=0.05, values must differ by 0.6755 log units or 4.73 fold (Appendix, page 71).
[d] n.d. indicates no data.

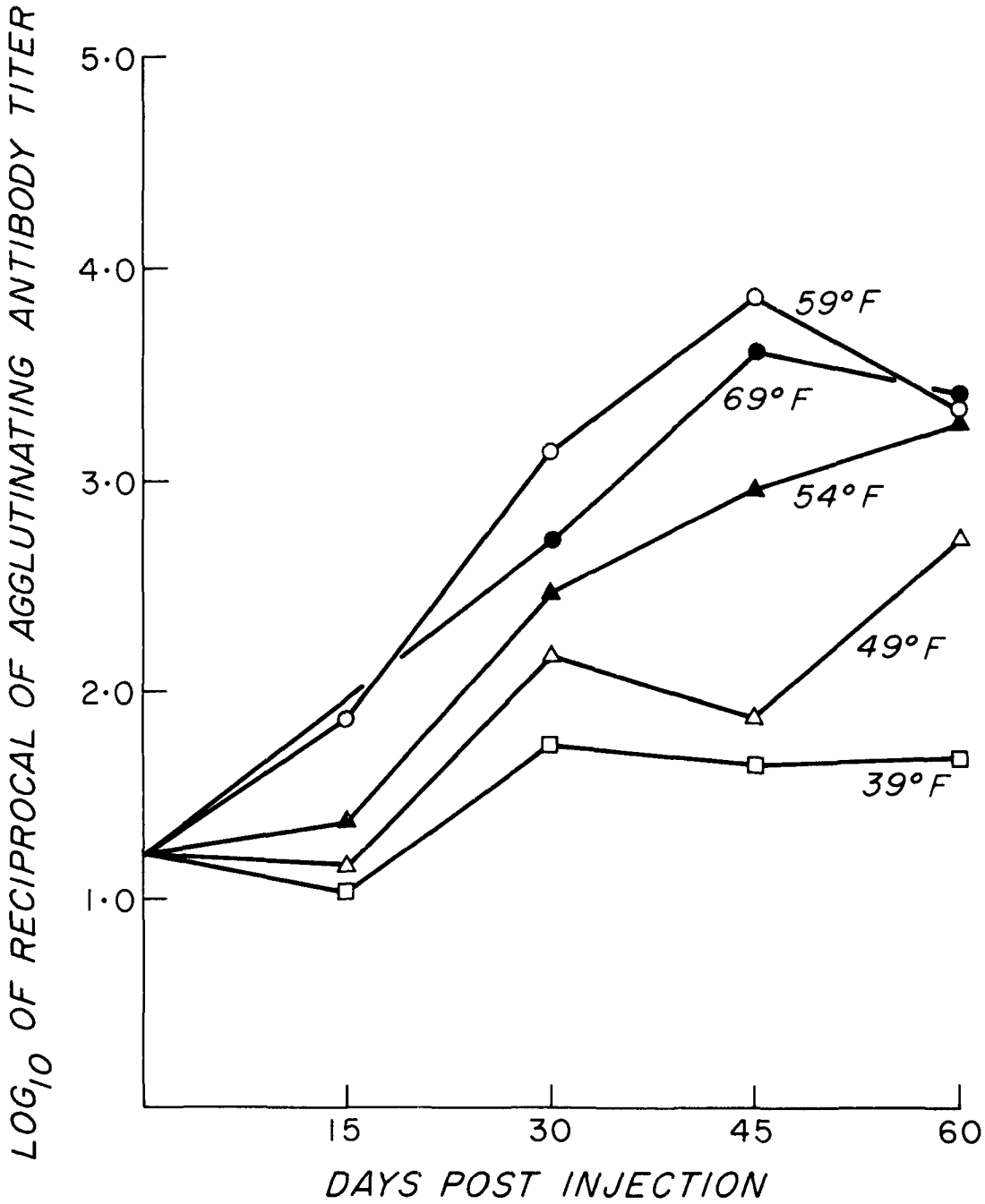

Fig. 8. Antibody response to an injection of killed Aeromonas salmonicida cells in juvenile coho salmon held at various water temperatures.

Antibody Response to Killed Kidney Disease Bacteria

At Various Water Temepratures

The results obtained in the experiment in which juvenile coho salmon received a single injection of killed kidney disease bacteria (*Corynebacterium sp.*) in Freund's adjuvant are shown in Table 13. Considerable mortality which was attributed to infection with *A. liquefaciens* occurred in the groups of fish held at 64, 69 and 74 F, and it was necessary to eliminate these groups from the experiment after 30 days. It is apparent that the results observed in the remaining temperature groups are quite different from those in the similar experiment with *A. salmonicida*. In this case the antibody response was much weaker at all water temperatures. The antibody titers at 30 days indicated the range from 44 to 54 F had permitted the formation of more antibody than lower or higher temperatures. However at 60 and 90 days after injection, no significant differences are apparent in the antibody levels from fish at 39 to 59 F. The data obtained in this experiment do not show significant effects of water temperature on antibody formation in response to a single injection of the causative agent of bacterial kidney disease. Rather, they indicate that this bacterium is a considerably weaker antigen than *A. salmonicida*.

After the final blood samples were collected at the 90 day period, the 25 vaccinated and 25 control fish in each temperature group from 39 to 59 F received a single intraperitoneal injection of about 9×10^8 living kidney disease bacteria. This challenge was intended to provide information concerning the degree of protection existing in the vaccinated fish held at the various temperatures. However this phase of the experiment was unsuccessful, as only 20 of the 250 fish that were injected with the living organisms succumbed within an observation period of 120 days. Eight of the 20 were from vaccinated groups and the remainder were controls.

Table 13. Agglutinating antibody levels in juvenile coho salmon following a single intraperitoneal injection of killed kidney disease bacteria in Freund's complete adjuvant.

Water Temperature	Post injection period in days	Reciprocals of agglutination titers			
		Vaccinated		Controls	
		Mean titer	Titer range	Mean titer	Titer range
54°F	0			< 20	< 20
39°F	15	34	20-80		< 20-20
	30	67	40-160		< 20-20
	45	160	160		< 20-20
	60	135	80-160		20-40
	75	113	80-160		< 20-20
	90	113	80-160		< 20-40
44°F	15	57	40-160		< 20-20
	30	269	160-320		20-40
	45	135	80-160		20-40
	60	135	80-160		20-40
	75	190	160-320		< 20-20
	90	95	80-160		< 20-40
49°F	15	40	20-80		< 20-20
	30	190	80-320		20
	45	113	80-160		< 20-40
	60	190	160-320		20-40
	75	135	80-160		< 20-40
	90	160	80-320		< 20-40
54°F	15	67	40-160		< 20-20
	30	269	160-320		< 20-20
	45	226	160-640		20-40
	60	160	80-640		< 20-40
	75	135	80-320		20-80
	90	113	80-160		< 20-20

Table 13 (continued). Agglutinating antibody levels in juvenile coho salmon following a single intraperitoneal injection of killed kidney disease bacteria in Freund's complete adjuvant.

Water Temperature	Post injection period in days	Reciprocals of agglutination titers			
		Vaccinated		Controls	
		Mean titer	Titer range	Mean titer	Titer range
59°F	15	57	40-80		< 20-20
	30	80	40-160		20-80
	45	160	80-320		20-80
	60	190	160-320		20-40
	75	269	160-320		20-80
	90	160	80-320		20-80
64°F[a]	15	32	<20-320		20
	30	95	20-320		< 20-20
69°F[a]	15	<20	<20-40		< 20-40
	30	28	20-40		< 20-20

[a]These groups of fish had to be eliminated from the experiment after 30 days because of mortality attributed to infection with A. liquefaciens.

SECTION IX

EFFECT OF WATER TEMPERATURE ON THE IMMUNE RESPONSE OF COHO SALMON TO ORAL VACCINATION WITH A KILLED *VIBRIO ANGUILLARUM* BACTERIN

Materials and Methods

The *Vibrio anguillarum* strain used in these experiments was isolated from coho salmon which died at Lint Slough, Waldport, Oregon, and was maintained on Cytophaga Sea water agar (Pacha and Ordal, 1967) at 4°C. Ten ml of tryptic soy broth were inoculated from a stock culture and incubated at 25 C for 12 hours. Two ml of this culture were then used to inoculate each of two one liter volumes of the same medium, which were also incubated for 12 hours. The resulting two liters of culture then served as the inoculum for a 30 liter volume of the same broth in a fermentor. After an incubation period of 10 to 12 hours, 500 ml of a 20% dextrose solution was added, and incubation continued for an additional 12 hours. Sufficient formalin was then added to give a final concentration of 0.3% formaldehyde. After one hour the cells were harvested in a Sharples continuous flow centrifuge, and the wet packed killed whole cells were frozen and stored at -26 C. For the oral immunization of fish the wet packed cells, after thawing, were incorporated in the Oregon Moist Pellet diet (8) in the proper proportion to give a final concentration of 2 mg of vaccine per gm of the ration.

Juvenile coho salmon were used in oral vaccination experiments. In the first experiment their average weight was 6.5 grams, and in a subsequent experiment smaller fish averaging 3.3 grams were used. Seven experimental water temperatures varying from 39 to 69 F at increments of 5 F were employed in the experiments. One or two groups of 75 to 100 fish were established at each of the seven temperatures, and the animals were gradually adjusted or tempered to their respective temperature levels by the method described in Section V.

Experimental Phase

When the tempering process was completed, oral immunization was begun. For a 15 day period, all groups of fish were fed Oregon Moist Pellet diet containing 2 mg of the wet whole cell vaccine per gram of ration. The diet was fed ad libitum, and because metabolism varies with temperature, the different groups of fish consumed varying amounts of the vaccine. The following table shows the amounts consumed by 100 fish averaging 6.5 grams in weight.

Table 14. Amount of diet and vaccine consumed in 15 days by groups of 100 coho salmon averaging 6.5 grams in weight, held at selected water temperatures.

Temperature at which fish were held	Total grams of diet consumed	Total milligrams of vaccine consumed
39 F	157	314
44 F	264	528
49 F	357	714
54 F	366	732
59 F	490	980
64 F	450	900
69 F	473	846

In the experiment with 3.3 gram fish, the amounts of diet and vaccine consumed were of course considerably smaller, but were influenced by temperature in a similar manner. During the immunization period a group of control fish were maintained on the same diet without vaccine at 54 F.

After the 15 day vaccination period all groups of fish were tempered back to 54 F. Water temperatures were changed by steps of 5 degrees F and fish were held at the new temperature for 48 hours before the next change was made. Thus tempering required a week for some groups. In the first experiment where 6.5 grams fish were used, after all the fish had been tempered back to 54 F they were held at this temperature for one week before being challenged by exposure to water containing virulent V. anguillarum. This was accomplished by transferring the fish to a salt water rearing impoundment at Lint Slough on the Oregon Coast. At this facility groups of the vaccinated fish were held in fiberglass

tanks one meter in diameter, supplied with salt water (about 3 gal. per min.) from the Slough. Vibrio anguillarum is constantly present in this water, and vibriosis reaches epizootic proportions among resident salmonids in the warmer months when water temperature rises above 54 F. The groups of vaccinated fish were held in the salt water at temperatures favorable for the progress of the disease (51 to 66 F) for 20 days. During this period fish that died were autopsied and cultures prepared by inoculating kidney tissue on brain heart infusion agar. Colonies that developed were examined by Gram stain and those that resembled V. anguillarum were confirmed by slide agglutination with specific antiserum.

The results of the first experiment are presented in Table 15. Very few deaths occurred in most of the vaccinated groups and in four of the seven groups, no deaths were shown to be due to vibriosis.

Among the unvaccinated controls, 44% died and 37% succumbed to infection with V. anguillarum. The degree of protection established by vaccination was not influenced by the water temperatures at which the fish were vaccinated. Those vaccinated at 39 F were protected just as well as the animals vaccinated at 69 F and in all groups vaccination was highly effective.

Additional data relating possible effects of water temperature on immunization was provided by two parallel experiments similar to the one described above but carried out concurrently. In these experiments the available coho salmon were smaller than those in the first experiment, having a mean weight of 3.3 grams. A further modification was made in the temperature at which the fish were held after vaccination. In the first experiment the fish were tempered back to 54 F and then held at that temperature for a week before being exposed to the infection in Lint Slough. In the subsequent pair of experiments, after completion of the 15 day vaccination period, each vaccinated group was held at its respective experimental temperature for one week before being tempered back to 54 F. The intent of this modification was to provide a somewhat longer period during which the various water temperatures might exert an effect on the immunization process. An experiment modified in this way might be more likely to reveal possible effects of temperature on immunization.

Except for the changes noted, the two parallel experiments were carried out in the same manner as the first one. The results obtained are shown in Table 16. Again, immunization was found to be highly effective at all experimental water temperatures. A small number of deaths from vibriosis occurred in the 39 and 44 F groups, compared with none in the 69 and 64 F groups, but the numbers are much too small to be significant. Thus the results confirm those in the first experiment and justify the conclusion that oral immunization of juvenile coho salmon with this V. anguillarum vaccine can be carried out effectively at any water temperatures in the range of 39 to 69 F.

Table 15. Effect of water temperature on the immune response in coho salmon to an orally administered bacterin of Vibrio anguillarum.

Temperature at Which Fish were Vaccinated[a]		Number of Fish/Group[b]	Total Number of deaths[c]	Number of Deaths Caused by V. anguillarum	Percent Mortality Caused by V. anguillarum
39 F	3.9 C	100	6	0	0
44 F	6.7 C	100	3	0	0
49 F	9.5 C	100	4	0	0
54 F	12.2 C	100	9	1	1
59 F	15.0 C	92	19	5	5
64 F	17.8 C	100	8	0	0
69 F	20.6 C	100	5	1	1
54 F	12.2 C Unvaccinated Control	100	44	37	37

[a]Vaccinated with 5 mg of vaccine/gm of Oregon Moist Pellets for 15 days followed by a 14 day tempering period.

[b]Mean weight 6.5 gm/fish.

[c]After 20 days natural challenge to V. anguillarum in salt water.

Table 16. Effect of water temperature on the immune response in coho salmon to an orally administered bacterin of Vibrio anguillarum

Temperature at Which Fish were Vaccinated[a]		Number of Fish/Group[b]	Total Number of deaths[c]	Number of Deaths Caused by V. anguillarum	Percent Mortality Caused by V. anguillarum
39 F	3.9 C	75	2	1	1
		100	13	5	5
44 F	6.7 C	95	4	2	2
		60	4	1	2
49 F	9.5 C	97	0	0	0
		100	6	2	2
54 F	12.2 C	90	2	0	0
		80	6	0	0
59 F	15.0 C	96	1	1	1
		100	0	0	0
64 F	17.8 C	97	0	0	0
		86	1	0	0
69 F	20.6 C	99	0	0	0
		87	0	0	0
54 F	12.2 C	100	72	72	72
	Unvaccinated Control	100	86	83	83

[a]Vaccinated with 5 mg of vaccine/gm of Oregon Moist Pellets for 15 days followed by a seven day tempering period.

[b]Mean weight 3.3 gm/fish.

[c]After 20 days natural challenge to V. anguillarum in salt water.

Discussion

The results of these experiments indicating that oral immunization of coho salmon with V. anguillarum vaccine is as effective at 39 or 44 F as at 59 to 69 F, on first consideration seem to conflict with the data reported in Section VIII concerning the effect of water temperature on the antibody response of coho salmon to an injection of killed cells of A. salmonicida. In the latter system water temperatures of 59 and 69 F were found to be optimal for antibody production. At these temperatures maximum levels of antibody were found after 45 days, while at 39 F very little antibody was present at this time. However, upon closer scrutiny, certain differences are evident in the two systems which may help to account for the apparent conflict in results. Perhaps the most important is the fact that in the A. salmonicida experiment, agglutinating antibody circulating in the blood was being measured, while in the oral immunization experiments with the vibrio vaccine, no agglutinating antibody was found in the blood. The mechanism by which oral immunity is produced has not yet been clarified. It could be due to formation of a secretory antibody localized in the intestinal tract, or possibly to a circulating antibody that is unable to cause agglutination but may cause lysis of the bacteria. Finally it might be a cellular type of immunity not associated with any antibody activity.

Since immunity against vibriosis does not require the presence of agglutinating antibody in the circulation, the effect of water temperature could well be different from its effect on agglutinating antibody for A. salmonicida. Another difference between the two systems that could influence the results lies in the fact that in the A. salmonicida experiment it was possible to maintain the fish at their respective temperatures throughout the entire experimental period, while in the V. anguillarum experiments it was necessary to temper the immunized fish back to 54 F before they could be transferred to the water of Lint Slough for challenge. This process required a week for some groups and it is possible that this brief period of exposure to warmer water could have enhanced the degree of immunity in the groups held at the lower temperatures. In the pair of experiments carried out concurrently this difficulty was minimized but was not eliminated entirely.

The data indicating the non-critical nature of water temperature in the range of 39 to 69 F in the process of immunization of juvenile salmon against vibriosis is also of some practical importance. This disease constitutes a serious threat to the success of marine aquaculture projects. An oral vaccine of the type described has now been tested rather extensively under controlled conditions and found to uniformly give a high degree of protection against the disease (9). The practical application of such a vaccine will be facilitated by the knowledge that it can be used effectively over a rather wide range of water temperatures.

SECTION X

ACKNOWLEDGMENTS

This project was supported by the Environmental Protection Agency over a two and one half year period beginning April 1, 1972 and ending September 30, 1974. The total funds provided by this agency amounted to $107,188. The assistance provided by Dr. Gerald R. Bouck, who served as Project Officer, is acknowledged with sincere thanks.

A major contribution to the project was made by the Fish Commission of Oregon and the Oregon Wildlife Commission. Without the support of these agencies none of the work described in this report would have been possible. They provided the large numbers of juvenile salmon and trout required for the experiments, without charge. The monetary value of the fish used during the two and one half year period of the project has been roughly estimated at about $7000.

SECTION XI

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SECTION XII

APPENDICES

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$ANOVA,4,3
$AVTABLE,8

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(   3) TEMP*EXPTYPE                   7  1.06616E 03   25.09
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 EXP              16    5.916304E 01

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5.400000E 01      4    4.000000E 01
5.900000E 01      4    3.300000E 01
6.400000E 01      4    4.200000E 01
6.900000E 01      4    4.901196E 01
7.200000E 01      4    4.891304E 01

 TEMP
    EXPTYPE

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4.900000E 01
     CON              2    6.000000E 00
     EXP              2    4.800000E 01
5.400000E 01
     CON              2    1.000000E 01
     EXP              2    7.000000E 01
5.900000E 01
     CON              2    1.000000E 01
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6.900000E 01
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7.200000E 01
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Analysis of Final Percent Mortality Data in Text Table 1. A. salmonicida in Chinook Salmon

Least significant difference 13.82% for P = 0.05

Least significant difference 19.04% for P = 0.01

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$AVTABLE,6

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(   3) TEMP*EXPTYPE                  7  1.13993E 03     19.49
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 TEMP

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5.4000000E 01       4    2.1000000E 01
5.9000000E 01       4    2.7000000E 01
6.4000000E 01       4    4.6000000E 01
6.9000000E 01       4    5.3000000E 01
7.4000000E 01       4    9.6000000E 01

 TEMP
    EXPTYPE

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     EXP               2    2.0000000E 00
4.4000000E 01
     CON               2          0E 00
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     CON               2    4.0000000E 00
     EXP               2    2.8000000E 01
5.4000000E 01
     CON               2    1.2000000E 01
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5.9000000E 01
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6.4000000E 01
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     EXP               2    8.4000000E 01
6.9000000E 01
     CON               2    1.0000000E 01
     EXP               2    9.6000000E 01
7.4000000E 01
     CON               2    9.6000000E 01
     EXP               2    9.6000000E 01

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Analysis of Final Percent Mortality Data in Text Table 3 . A. salmonicida in Steelhead Trout

Least significant difference 16.22% for P = 0.05

Least significant difference 22.34% for P = 0.01

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$AVTABLE,B

ANALYSIS OF VARIANCE FOR PMORT
 LINE   SOURCE OF VARIATION          DF   MEAN SQUARE        F
(   1) TEMP                           7   7.279399E 02      9.77
(   2) EXPTYPE                        1   9.940500E 03    133.43
(   3) TEMP*EXPTYPE                   7   1.037244E 03     13.93
(   4) ERROR                         16   7.465000E 01
       TOTAL                         31

$INTERMEANS,3,4,3*4

 EXPTYPE

    VALUE        FREQ   MEAN OF PMORT
 CON               16    5.750000E 00
 EXP               16    4.100000E 01

 TEMP

    VALUE        FREQ   MEAN OF PMORT
3.900000E 01        4    1.300000E 01
4.400000E 01        4    1.300000E 01
4.900000E 01        4    2.000000E 00
5.400000E 01        4    2.000000E 01
5.900000E 01        4    2.900000E 01
6.400000E 01        4    3.400000E 01
6.900000E 01        4    3.500000E 01
7.200000E 01        4    4.100000E 01

 TEMP
    EXPTYPE

    VALUE            FREQ   MEAN OF PMORT
3.900000E 01
     CON                2    1.400000E 01
     EXP                2    1.200000E 01
4.400000E 01
     CON                2    1.200000E 01
     EXP                2    1.400000E 01
4.900000E 01
     CON                2    4.000000E 00
     EXP                2          0E 00
5.400000E 01
     CON                2    2.000000E 00
     EXP                2    3.800000E 01
5.900000E 01
     CON                2    2.000000E 00
     EXP                2    5.600000E 01
6.400000E 01
     CON                2    6.000000E 00
     EXP                2    6.200000E 01
6.900000E 01
     CON                2    2.000000E 00
     EXP                2    6.800000E 01
7.200000E 01
     CON                2    4.000000E 00
     EXP                2    7.800000E 01

$END
```

Analysis of Final Percent Mortality Data in Text Table 5. A. liquefaciens in Chinook Salmon.

Least significant difference 13.30% for P = 0.05.

Least significant difference 25.21% for P = 0.01.

```
$NAME,3 EXPTYPE,4 TEMP,5 A,6 B,8 PMORT
$SET,8=(A/3)*100.
$ORDER,4,3,8,(4,3)
$ANOVA,4,3
$AVTABLE,8

ANALYSIS OF VARIANCE FOR PMORT
 LINE  SOURCE OF VARIATION          DF  MEAN SQUARE      F
(   1) TEMP                          7  2.456566E 03   437.72
(   2) EXPTYPE                       1  2.389675E 04  4256.21
(   3) TEMP*EXPTYPE                  7  2.346795E 03   418.16
(   4) ERROR                        16  5.61225E 00
       TOTAL                        31

$INTERMEANS,3,4,3*4

 EXPTYPE

    VALUE        FREQ   MEAN OF PMORT
 CON                16    5.357143E-01
 EXP                16    5.517857E 01

 TEMP

    VALUE        FREQ   MEAN OF PMORT
3.9000000E 01       4              0E 00
4.4000000E 01       4              0E 00
4.9000000E 01       4    1.428571E 00
5.4000000E 01       4    2.071429E 01
5.9000000E 01       4    4.857143E 01
6.4000000E 01       4    5.071429E 01
6.9000000E 01       4    5.142857E 01
7.4000000E 01       4    5.000000E 01

 TEMP
    EXPTYPE

    VALUE          FREQ   MEAN OF PMORT
3.9000000E 01
     CON               2              0E 00
     EXP               2              0E 00
4.4000000E 01
     CON               2              0E 00
     EXP               2              0E 00
4.9000000E 01
     CON               2              0E 00
     EXP               2    2.857143E 00
5.4000000E 01
     CON               2              0E 00
     EXP               2    4.142857E 01
5.9000000E 01
     CON               2              0E 00
     EXP               2    9.714286E 01
6.4000000E 01
     CON               2    1.428571E 00
     EXP               2    1.000000E 02
6.9000000E 01
     CON               2    2.857143E 00
     EXP               2    1.000000E 02
7.4000000E 01
     CON               2              0E 00
     EXP               2    1.000000E 02

$END
```

Analysis of Final Percent Mortality Data in Text Table 7 . A. liquefaciens in Coho Salmon

Least significant difference for P = 0.05. **5.02%**

Least significant difference for P = 0.01 **6.92%**

PROBLEM I-D-COL-1

ANALYSIS OF VARIANCE FOR PMORT

LINE	SOURCE OF VARIATION	DF	MEAN SQUARE	F
(1)	TEMP	7	5.04392E 03	325.42
(2)	EXPTYPE	1	7.81250E 03	504.03
(3)	TEMP*EXPTYPE	7	1.68564E 03	108.75
(4)	ERROR	16	1.55000E 01	
	TOTAL	31		

SOURCE MEANS

TYPE

(1)	(2)
45.75000	14.50000

TEMP

(1)	(2)	(3)	(4)
100.000	51.000	51.000	28.000
(5)	(6)	(7)	(8)
10.000	0	1.000	0

TYPE X TEMP (TEMP VARIES MOST REPIDLY.)

100.000	100.000	92.000	56.000
16.000	0	2.000	0
100.000	2.000	10.000	0
4.000	0	0	0

Analysis of Final Percent Mortality Data
in Text Table 9 . Columnaris Disease
in Steelhead Trout.

Least significant difference 8.21%
for P = 0.05
Least significant difference 11.31%
for P = 0.01

```
$NAME,3 EXPTYPE,4 TEMP,6 8,7 0,4 PMORT
$SET,8=(0/3)*100.
$ORDER,4,1,8,(+,3)
$ANOVA,4,3
$AVTABLE,8

ANALYSIS OF VARIANCE FOR PMORT
 LINE  SOURCE OF VARIATION          DF  MEAN SQUARE      F
(   1) TEMP                          6  1.09317E 03    12.35
(   2) EXPTYPE                       1  3.31851E 04   374.92
(   3) TEMP*EXPTYPE                  6  1.02000E 03    11.52
(   4) ERROR                        14  8.85131E 01
       TOTAL                        27

$INTERMEANS,3,4,3*4

 EXPTYPE

    VALUE        FREQ   MEAN OF PMORT
 CON                14    5.962733E-01
 EXP                14    6.944024E 01

 TEMP

    VALUE        FREQ   MEAN OF PMORT
3.900000E 01        4    3.225684E 01
4.400000E 01        4    4.773913E 01
4.900000E 01        4    5.000000E 01
5.400000E 01        4    5.000000E 01
5.900000E 01        4    3.800000E 01
6.400000E 01        4    2.045455E 01
6.900000E 01        4    6.728778E 00

 TEMP
    EXPTYPE

    VALUE            FREQ   MEAN OF PMORT
3.900000E 01
     CON                2          0E 00
     EXP                2    6.447368E 01
4.400000E 01
     CON                2    2.000000E 00
     EXP                2    9.347826E 01
4.900000E 01
     CON                2    2.173913E 00
     EXP                2    9.782609E 01
5.400000E 01
     CON                2          0E 00
     EXP                2    1.000000E 02
5.900000E 01
     CON                2          0E 00
     EXP                2    7.600000E 01
6.400000E 01
     CON                2          0E 00
     EXP                2    4.090909E 01


6.900000E 01
     CON                2          0E 00
     EXP                2    1.345756E 01
```

Analysis of Final Percent Mortality Data in Text Table 10. Kidney Disease in Coho Salmon.

Least significant difference 20.18% for P= 0.05.
Least significant difference 28.01% for P=0.01.

```
$VAR,10
$NOMINAL,1,3
$READ,*STEEK1,1-7
$NAME,3 EXPTYPE,4 TEMP,6 B,7 C,8 PMORT
$SET,8=(C/9)*100.
$ORDER,4,3,8,(4,3)
$ANOVA,4,3
$AVTABLE,8

ANALYSIS OF VARIANCE FOR PMORT
 LINE   SOURCE OF VARIATION          DF   MEAN SQUARE      F
(   1)  TEMP                          6   1.06224E 03    20.80
(   2)  EXPTYPE                       1   2.29393E 04   449.12
(   3)  TEMP*EXPTYPE                  6   1.06224E 03    20.80

(   4)  ERROR                        14   5.10764E 01

        TOTAL                        27

$INTERMEANS,3,4,3*4

 EXPTYPE

    VALUE        FREQ   MEAN OF PMORT
 CON                14            0E 00
 EXP                14    5.724543E 01

 TEMP

    VALUE        FREQ   MEAN OF PMORT
3.900000E 01        4    1.726316E 01
4.400000E 01        4    4.900000E 01
4.900000E 01        4    4.522222E 01
5.400000E 01        4    3.887363E 01
5.900000E 01        4    2.453704E 01
6.400000E 01        4    2.129630E 01
6.900000E 01        4    4.166667E 00

 TEMP
    EXPTYPE

    VALUE            FREQ   MEAN OF PMORT
3.900000E 01
     CON                2            0E 00
     EXP                2    3.452632E 01
4.400000E 01
     CON                2            0E 00
     EXP                2    9.800000E 01
4.900000E 01
     CON                2            0E 00
     EXP                2    9.044444E 01
5.400000E 01
     CON                2            0E 00
     EXP                2    7.774725E 01
5.900000E 01
     CON                2            0E 00
     EXP                2    4.907407E 01
6.400000E 01
     CON                2            0E 00
     EXP                2    4.259259E 01
6.900000E 01
     CON                2            0E 00
     EXP                2    9.333333E 00

$END
```

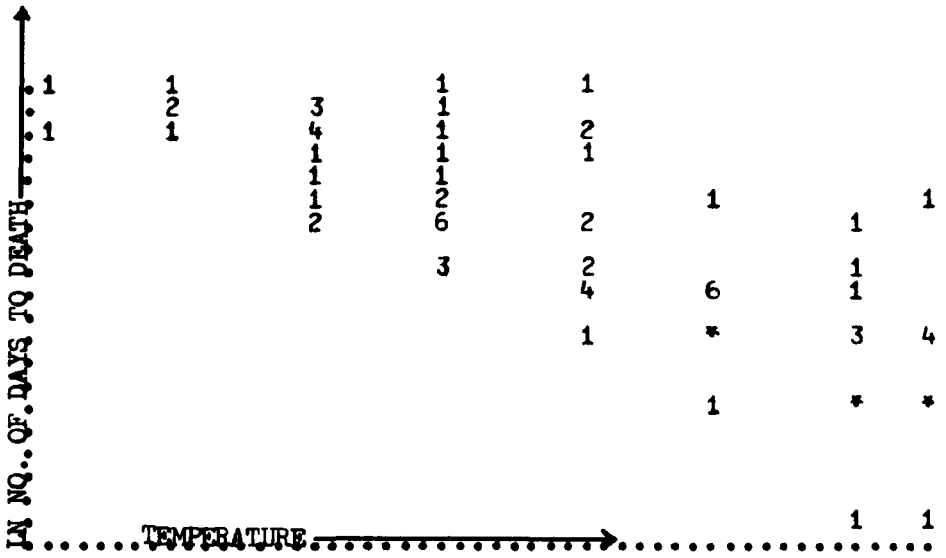
Analysis of Final Percent Mortality Data in Text Table //. Kidney Disease in Steelhead Trout.

Least significant difference 15.33% for P = 0.05

Least significant difference 21.28% for P = 0.01

ANALYSIS OF DAILY MORTALITY DATA USED IN COMPUTING MEAN VALUES SHOWN IN TEXT FIG. 7. A. SALMONICIDA IN CHINOOK SALMON

```
*SIPS LOG UNIT: 12/16/74   2:52 PM
$VAR,3
$READ,*CHINS21,1-2
$SET,3=LN(2)
$NAME,1 TEMP,2 DAYS,3 LNDAYS
$SCATTER,TEMP,LNDAYS
LOWER BOUND OF X:      3.90000E 01
UPPER BOUND OF X:      7.20000E 01
LOWER BOUND OF Y:            0E 00
UPPER BOUND OF Y:      2.70805E 00
CORRELATION COEFFICIENT = -0.8606855
```




```
$REGRESS,LNDAYS ON TEMP
LNDAYS  = 1.4134E+00
:ADD,TEMP

LNDAYS  = 5.4010E+00          -6.4613E-02 TEMP
:AVTABLE

            ANALYSIS OF VARIANCE TABLE

SOURCE       DF    SUM OF SQUARES          MEAN SQUARE
TOTAL       108    4.99565192E 01      4.62560363E-01

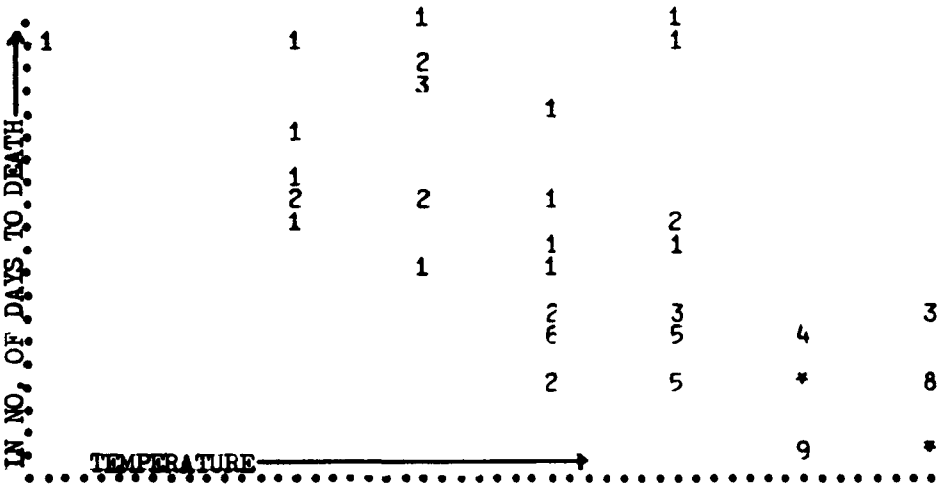
REGRESSION    1    3.70067652E 01      3.70067652E 01

RESIDUAL    107    1.29497539E 01      1.21025738E-01

              R SQUARED =    .74077950
```

ANALYSIS OF DAILY MORTALITY DATA USED IN COMPUTING MEAN VALUES SHOWN IN TEXT FIG. 2. A. SALMONICIDA IN STEELHEAD TROUT.

```
*SIPS LOG UNIT: 12/17/74  11:43 AM
$VAR,3
$READ,*STEES21,1-2
$SET,3=LN(2)
$NAME,1 TEMP,2 DAYS,3 LNDAYS
$SCATTER,TEMP,LNDAYS
LOWER BOUND OF X:      3.90000E 01
UPPER BOUND OF X:      7.40000E 01
LOWER BOUND OF Y:      6.93147E-01
UPPER BOUND OF Y:      3.36730E 00
CORRELATION COEFFICIENT = -0.7635207
```




```
$REGRESS,LNDAYS ON TEMP
LNDAYS  = 1.4698E+00
:ADD,TEMP

LNDAYS  = 6.0565E+00          -7.0690E-02 TEMP
:AVTABLE

            ANALYSIS OF VARIANCE TABLE

SOURCE        DF    SUM OF SQUARES          MEAN SQUARE
TOTAL         95    5.27143243E 01      5.54887624E-01
REGRESSION     1    3.07305437E 01      3.07305437E 01
RESIDUAL      94    2.19837806E 01      2.33870007E-01
              R SQUARED =    .58296382
```

ANALYSIS OF DAILY MORTALITY DATA USED IN COMPUTING MEAN VALUES SHOWN IN TEXT FIG. 3 A. LIQUEFACIENS IN COHO SALMON.

```
*SIPS LOG UNIT: 01/07/75   1:17 PM

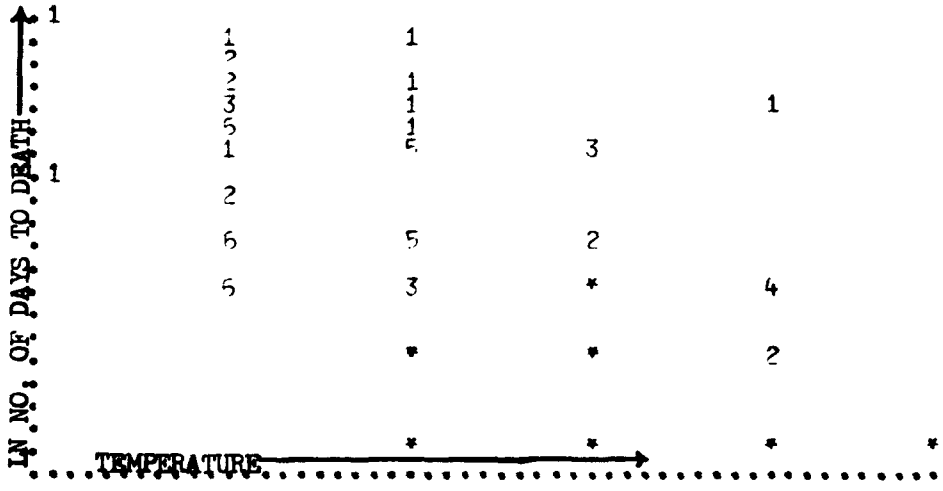
$READ,*COHOL21,1-2

$APPEND,*COHOL22,1-2

$SET,?=LN(2)

$SCATTER,TEMP,LNDAYS
LOWER BOUND OF X:      4.90000E 01
UPPER BOUND OF X:      7.40000E 01
LOWER BOUND OF Y:            0E 00
UPPER BOUND OF Y:      2.89037E 00

CORRELATION COEFFICIENT = -0.7016998
```




```
$REGRESS,LNDAYS ON TEMP

LNDAYS  = 4.7780E-01


:ADD,TEMP

LNDAYS  = 5.4592E+00          -7.6300E-02 TEMP

:AVTABLE

                ANALYSIS OF VARIANCE TABLE

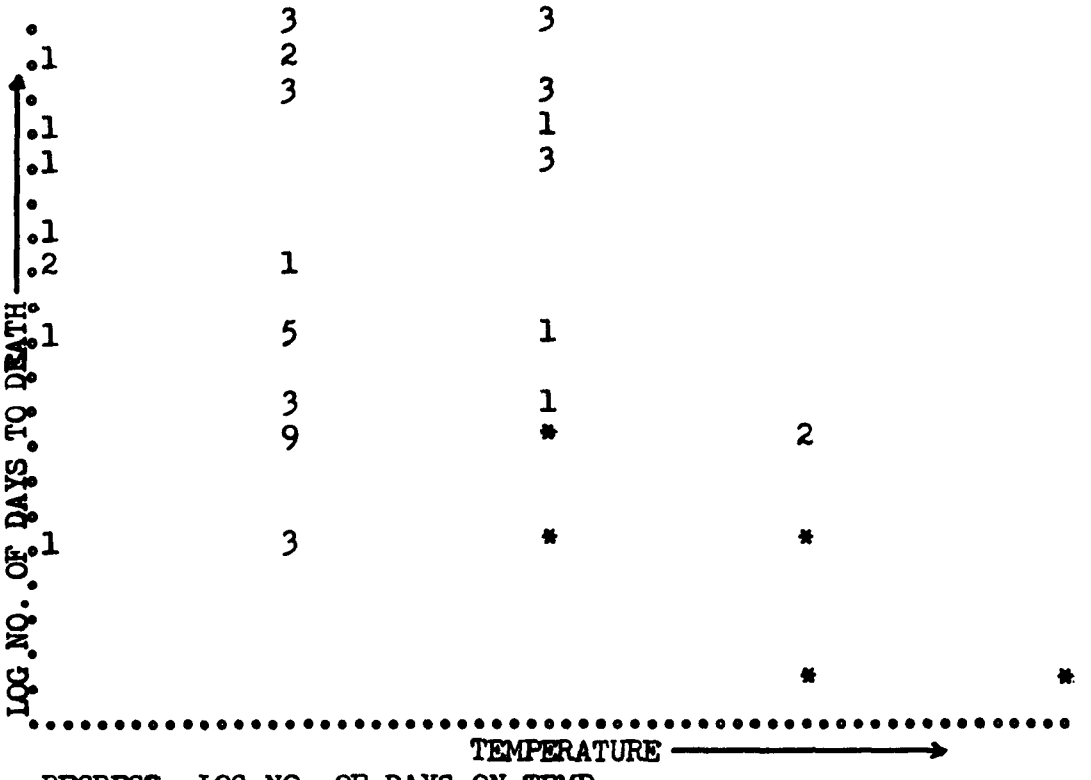
SOURCE          DF    SUM OF SQUARES          MEAN SQUARE
TOTAL          309    1.57508378E 02      5.09737792E-01

REGRESSION       1    7.75546795E 01      7.75546795E 01

RESIDUAL       308    7.99542984E 01      2.59591878E-01

                R SQUARED =    .49238260
```

ANALYSIS OF DAILY MORTALITY DATA USED IN COMPUTING MEAN VALUES SHOWN IN TEXT FIG. 5. COLUMNARIS DISEASE IN STEELHEAD TROUT.



REGRESS, LOG NO. OF DAYS ON TEMP

LOG NO. OF DAYS = 3.5715E - 01

: ADD, TEMP

LOG NO. OF DAYS = 3.6361E+00 -4.9044E - 02 TEMP

ANALYSIS OF VARIANCE TABLE

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
TOTAL	181	2.60560919E 01	1.43956309E -01
REGRESSION	1	1.50676135E 01	1.50676135E -01
RESIDUAL	180	1.09884783E 01	6.10471017E -02

R SQUARED = .57827604

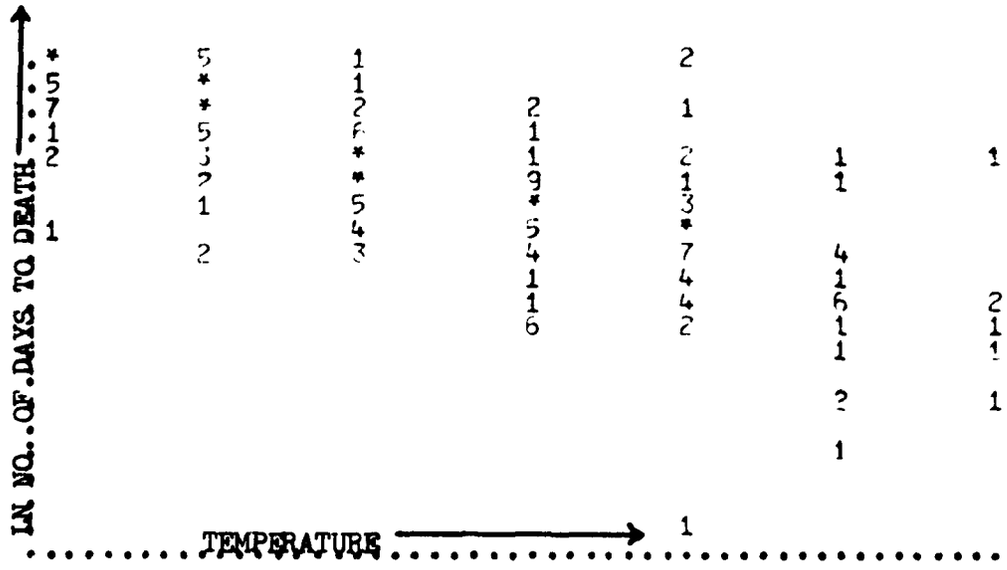
CORRELATION COEFFICIENT = - 0.7604

ANALYSIS OF DAILY MORTALITY DATA USED IN COMPUTING MEAN VALUES SHOWN IN TEXT FIG. 6 . KIDNEY DISEASE IN COHO SALMON.

```
*STPS LOG UNIT: 01/07/75  12:50 PM

$SCATTER,TEMP,LNDAYS
LOWER BOUND OF X:        3.000000E 01
UPPER BOUND OF X:        6.900000E 01
LOWER BOUND OF Y:        1.791759E 00
UPPER BOUND OF Y:        4.663439E 00

CORRELATION COEFFICIENT = -0.7496363
```




```
$REGRESS,LNDAYS ON TEMP
LNDAYS  = 3.8352E+00
:ADD,TEMP

LNDAYS  = 5.3019E+00          -4.8024E-02 TEMP
:AVTABLE

                ANALYSIS OF VARIANCE TABLE

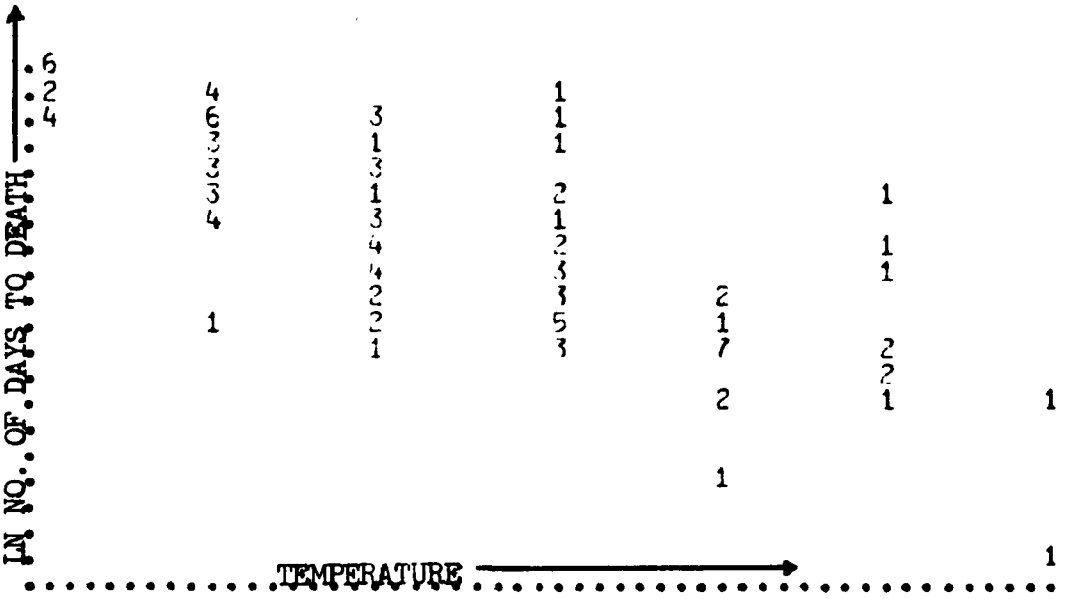
SOURCE          DF    SUM OF SQUARES          MEAN SQUARE
TOTAL          217    5.6229453?E 01       2.59260155E-01
REGRESSION       1    3.16152565E 01       3.16152565E 01
RESIDUAL       216    2.46441972E 01       1.14093505E-01
                R SQUARED =    .56195456
```

ANALYSIS OF DAILY MORTALITY DATA USED IN COMPUTING MEAN VALUES SHOWN IN TEXT FIG. 7 . KIDNEY DISEASE IN STEELHEAD TROUT.

```
$SCATTER,TEMP,LNDAYS
LOWER BOUND OF X:        3.90000E 01
UPPER BOUND OF X:        6.90000E 01
LOWER BOUND OF Y:        1.94591E 00
UPPER BOUND OF Y:        4.65396E 00

CORRELATION COEFFICIENT = -0.7927331

NUMBER OF MISSING OBSERVATIONS =      11
```




```
$REGRESS,LNDAYS ON TEMP

LNDAYS  = 3.7183E+00

$ADD,TEMP

LNDAYS  = 6.6657E+00          -5.8337E-02 TEMP

$AVTABLE

                ANALYSIS OF VARIANCE TABLE

SOURCE          DF    SUM OF SQUARES          MEAN SQUARE
TOTAL          104    3.2525653 6E 01      3.12746669E-01

REGRESSION       1    2.0439960 6E 01      2.0439960 6E 01

RESIDUAL       103    1.2085693 0E 01      1.1733682 6E-01

                R SQUARED =    .62842582
```

ANALYSIS OF THE REPLICATE ANTIBODY TITERS IN APPENDIX TABLE A TO DETERMINE THE LEAST SIGNIFICANT DIFFERENCE BETWEEN MEAN ANTIBODY TITERS IN TEXT TABLE 12 AND FIG. 8

Twenty nine sets of 5 replicate antibody titers from appendix Table A were selected for the analysis. Fifteen represented serum samples from vaccinated fish and 14 were titers from unvaccinated controls. The 15 sets of data from vaccinated fish included those showing the greatest degrees of variation among the 5 replicates in each set.

All of the titers were first transformed to $\log_{10}$ values, and the mean of each set of 5 replicates was determined. Deviations from the mean were then calculated for each value in each set; each deviation was then squared and the sum of all these squares determined.

Sum of squares of deviations from mean values = 32.9823

Divisor (sum of degrees of freedom) or (5-1) x 29 = 116

$$\text{Variance} = \frac{32.9823}{116} = 0.28433$$

$$\text{Estimated standard deviation} = \sqrt{0.28433} \quad 0.53322$$

$$\text{Standard error of difference between means} \quad \sqrt{\left(\frac{0.53322}{5}\right)^2 \times 2} = \frac{0.53322}{2.23606} \times 1.414 = 0.33718$$

With 116 degrees of freedom and P = 0.05, t = 2.0

Least significant difference = 2.0 x 0.33718 = 0.67436 = $\log_{10}$ of 4.73

Hence 2 mean titer values in test Table 12 must differ by 0.67436 log units, or 4.73 fold to be considered significantly different.

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO.	2.	3. RECIPIENT'S ACCESSION NO.
EPA-600/3-76-021		

4. TITLE AND SUBTITLE	5. REPORT DATE
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	6. PERFORMING ORGANIZATION CODE: NA

7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
J.L. FRYER, K.S. PILCHER, J.F. SANDERS, J.S. ROHOVEC, I.I. ZINN, W.J. GROBERG, AND R.H. McCOY	NA

9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
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15. SUPPLEMENTARY NOTES

16. ABSTRACT

Investigations of the effect of temperature on infections of salmonid fish were conducted. Aeromonas salmonicida infection was studied in chinook salmon and steelhead trout; Aeromonas liquefaciens infection was studied in chinook and coho salmon. In all cases, mortality rates were high at 64 to 69 F; usually moderate at 54 to 59 F; and low or zero at 39 to 49 F. Progress of the infections was accelerated at higher temperatures and retarded at lower temperatures. Bacterial kidney disease was studied in choh salmon and steelhead trout. The temperature range of 44 to 54 was optimal for the development of fatal infection as indicated by mortality rates of 78 to 100%. Higher temperatures had a suppressing effect, which was marked at 69 F. Temperatures of 59 to 69 F were optimal for the formation of agglutinating antibody when juvenile choh salmon were injected with a killed suspension of A. salmonicida. At lower temperatures less antibody was formed and no significant amount was produced at 39 F 60 days after injection of antigen. Oral immunization of juvenile coho salmon with a vaccine consistedof formalin killed Vibrio anguillarum cells incorporated in their diet , protected them against fatal infection when the fish were held at temperatures from 39 to 69 during immunization.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
SALMON, TROUT, ENVIRONMENTS, PATHOPHYSIOLOGY WATER POLLUTION, WASTE WATER, FRESHWATER DISEASES, ANTIBODIES	THERMAL POLLUTION	06/c/F/M/S/T

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